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University of Alberta

MICROBIAL METABOLISM OF CARBAZOLE AND OTHER COMPONENTS OF COAL TAR CREOSOTE

by

LISA MARIE GIEG ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Microbiology

Edmonton, Alberta

Spring 1996

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Lisa Gieg

11432 71 Avenue
Edmonton, Alberta
T6G 0A7

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled MICROBIAL METABOLISM OF CARBAZOLE AND OTHER COMPONENTS OF COAL TAR CREOSOTE submitted by LISA MARIE GIEG in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

P Fedorak
Dr. P.M. Fedorak, Supervisor

Dr. M.A. Pickard

Dr. D.W.S. Westlake

Dr. W.J. Page

Dr. W.B. McGill

Dr. X.C. Le

Dr. E.L. Madsen
Cornell University

November 8, 1995

University of Alberta
Edmonton

Canada T6G 2E9

Department of Biological Sciences
Faculty of Science

CW 405 Biological Sciences Centre
Telephone: Office (403) 492-3308
Fax (403) 492-9234
Chair (403) 492-0672

December 8, 1995

Lisa Gieg
Department of Biological Sciences
Universtiy of Alberta
T6G 2E9

Dear Ms. Gieg:

We grant you permission to include portions of our co-authored manuscript, entitled "Carbazole Degradation by *Pseudomonas* sp. LD2: Metabolic Characteristics and the Identification of Some Metabolites" in your thesis chapter on carbazole degradation.

Sincerely,

P Fedorak

P.M. Fedorak, Ph.D.
Dept. Biological Sciences

A Otter

A. Otter, Ph.D.
Dept. Chemistry

TO MY FAMILY AND FRIENDS

In appreciation

of their

love, laughter, and inspiration

ABSTRACT

Three different aspects of the microbial degradation of coal tar creosote were studied as part of a larger 5-year field experiment examining the fate of aromatic compounds in a well-characterized creosote-contaminated aquifer near Borden, Ontario, Canada.

In the first of these studies, the general biodegradability of aromatic compounds in a commercial coal tar creosote (OCT) was examined by establishing seven creosote-degrading populations from different sources as shake flask cultures. The seven populations removed creosote chemicals to different extents, with the greatest extent of removal of 3- and 4-ringed aromatic compounds observed in cultures originally enriched from creosote-contaminated soils.

Another aspect of research involved determining whether the Borden aquifer material had microbial iron-reducing activity, which was measured by monitoring Fe(II) production. Borden aquifer material was found to contain microbial iron-reducing populations because an increase in Fe(II) production was observed when live sediments were incubated with several different carbon sources, including water-soluble OCT compounds. Increases in Fe(II) production were not seen when the sediments were heat-killed.

The main focus of this research was the aerobic, microbial metabolism of carbazole. A carbazole-degrading bacterium, *Pseudomonas* sp. LD2, was isolated by enrichment from creosote-contaminated soil and utilized carbazole as a sole source of carbon, nitrogen, and energy. Several aromatic and heterocyclic compounds were tested as growth substrates for isolate LD2 but few supported the growth of isolate LD2. Anthranilic acid and catechol served as growth substrates and were positively identified as intermediates of carbazole degradation, along with *cis, cis*-muconic acid, by isolate LD2. In addition, eleven nitrogen-containing metabolites were observed in diazomethane-derivatized acidified

extracts of LD2 culture supernatants by gas chromatography (GC), four of which were unequivocally identified using GC-mass spectrometry or NMR spectroscopy. These included indole-3-acetic acid, 5-(2-aminophenyl)-5-oxo-pentanoic acid, and the cyclized products of 5-(2-aminophenyl)-5-oxo-penta-3-enoic acid and 6-(2-aminophenyl)-2-hydroxy-6-oxo-hexa-2,4-dienoic acid. Based on these findings, a pathway for carbazole degradation by isolate LD2 is proposed. Several of these metabolites were also observed in supernatant extracts of mixed carbazole-degrading populations.

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1. INTRODUCTION 1
1.1 Aims of the Borden project 1
1.2 The Borden aquifer and emplacement of creosote 1
1.3 Field and laboratory studies 2
1.4 Literature Cited 3
2. LITERATURE REVIEW 5
2.1 Creosote as a wood preservative and extent of creosote contamination 5
2.2 Chemical composition of creosote 6
2.3 Health risks associated with compounds in creosote 6
2.3.1 Phenols 6
2.3.2 PAHs and NSO compounds 8
2.4 Fate of creosote chemicals in the environment 9
2.5 Biodegradation of creosote compounds 12
2.6 Aerobic degradation of creosote compounds 13
2.6.1 Phenols 13
2.6.2 Polycyclic aromatic hydrocarbons 13
2.6.3 Nitrogen-, sulfur-, and oxygen-heterocycles 19
2.6.3.1 Sulfur-heterocycles 19
2.6.3.2 Oxygen-heterocycles 19
2.6.3.3 Nitrogen-heterocycles 20
2.7 Anaerobic degradation of creosote compounds 25
2.7.1 Phenols 27
2.7.2 Polycyclic aromatic hydrocarbons 29
2.7.3 Nitrogen-, sulfur-, and oxygen-heterocycles 29
2.8 Iron reduction 31
2.8.1 Environmental significance of microbial iron reduction 32
2.8.2 Effect of iron form on dissimilatory iron reduction 33
2.8.3 Substrates for iron-reducers 34
2.8.3.1 Fermentable substrates 34
2.8.3.2 Organic acids and hydrogen 34
2.8.3.3. Aromatic compounds 35
2.8.4 *Geobacter* and other dissimilatory iron-reducers 37
2.9 Bioremediation of creosote-contaminated sites 38
2.9.1 The beginning of bioremediation 39
2.9.2 Bioremediation technologies 40
2.9.2.1 Solid-phase bioremediation 40

2.9.2.2 Slurry-phase bioremediation 41
2.9.2.3 In situ bioremediation 42
2.9.2.4 Choice of bioremediation strategy 43
2.9.3 Creosote-contaminated sites 44
2.10 Research Objectives 46
2.11 Literature Cited 47
3. THE AEROBIC BIODEGRADATION OF COAL TAR CREOSOTE 58
3.1 INTRODUCTION 58
3.2 MATERIALS AND METHODS 61
3.2.1 Characterization of creosotes used 61
3.2.2 General culturing methods 62
3.2.2.1 Initial enrichments on Sidney coal tar 62
3.2.2.2 Enrichments on Ontario coal tar creosote 63
3.2.3 Extractions of cultures 64
3.2.4 Experiments with radiolabelled substrates 65
3.2.5 Plating of cultures 66
3.2.6 Anaerobic experiments with OCT 66
3.2.7 Analytical methods 67
3.3 RESULTS 68
3.3.1 Sidney coal tar 68
3.3.2 Ontario coal tar creosote (OCT) 70
3.3.2.1 Chemical analysis of OCT 70
3.3.2.2 Qualitative OCT analysis 71
3.3.2.3 Extent of removal of compounds in OCT 75
3.3.2.3.1 OCT as the sole carbon source 75
3.3.2.3.2 OCT, carbazole, and phenol as carbon sources 77
3.3.3 Removal of OCT compounds at different incubation temperatures 81
3.3.4 Mineralization of compounds in creosote 83
3.3.5 Characterization of creosote-degrading populations 83
3.3.5.1 Degradation of aromatic hydrocarbons on agar plates 83
3.3.5.2 Mineralization of saturated and aromatic hydrocarbons 85
3.3.6 Anaerobic experiments 88

- 3.4 DISCUSSION 90
- 3.5 LITERATURE CITED 98

4. IRON REDUCTION ACTIVITY IN BORDEN AQUIFER SEDIMENTS 102

- 4.1 INTRODUCTION 102
- 4.2 MATERIALS AND METHODS 105
  - 4.2.1 General anaerobic techniques 106
  - 4.2.2 Media for iron-reducers 106
    - 4.2.2.1 Description of media 106
    - 4.2.2.2 Synthesis of ferric oxyhydroxide 107
    - 4.2.2.3 Iron chelators 107
  - 4.2.3 Substrates 108
    - 4.2.3.1 Aromatic and non-aromatic compounds 108
    - 4.2.3.2 Water-soluble creosote compounds 108
  - 4.2.4 Organisms and inocula used for iron reduction studies 109
    - 4.2.4.1 *Geobacter metallireducens* 109
    - 4.2.4.2 Borden aquifer material 109
      - 4.2.4.2.1 Sources of cores and amount of Fe(III) 109
      - 4.2.4.2.2 Culturing of Borden core material 110
  - 4.2.5 Iron assays 111
    - 4.2.5.1 Measurement of Fe(II) production 111
    - 4.2.5.2 Measurement of microbially-reducible Fe(III) 111
  - 4.2.6 Substrate analyses 112
    - 4.2.6.1 Acetate, propionate, and butyrate analysis 112
    - 4.2.6.2 Aromatic substrate analysis 112
    - 4.2.6.3 Aqueous OCT composition 113
- 4.3 RESULTS 113
  - 4.3.1 Experiments with aerobic core samples 113
    - 4.3.1.1 Aerobic core #1 113
    - 4.3.1.2 Aerobic core #2 115
  - 4.3.2 Experiments with anaerobic core material 118
    - 4.3.2.1 Experiments with non-aromatic and aromatic substrates 118
    - 4.3.2.2 Experiments with water-soluble OCT components 121
- 4.4 DISCUSSION 128
- 4.5 LITERATURE CITED 138

5. AEROBIC BIODEGRADATION OF CARBAZOLE, A NITROGEN-HETEROCYCLE FOUND IN COAL TAR CREOSOTE 142
- 5.1 INTRODUCTION 142
- 5.2 MATERIALS AND METHODS 144
  - 5.2.1 Growth media and conditions 144
  - 5.2.2 Enrichments for carbazole-degrading populations 145
  - 5.2.3 Isolation of a carbazole-degrading bacterium 145
  - 5.2.4 Transposon mutants of LD2 147
  - 5.2.5 Substrates tested for the growth of isolate LD2 148
  - 5.2.6 Mineralization of radiolabelled carbazole and other substrates 149
  - 5.2.7 Extraction of metabolites from carbazole-degrading cultures 150
  - 5.2.8 Derivatization methods 150
  - 5.2.9 Washed cell suspension experiments 151
  - 5.2.10 Enzyme assays 152
  - 5.2.11 Purification of carbazole metabolites by TLC 153
  - 5.2.12 Chemical syntheses 153
  - 5.2.13 Analytical methods 154
- 5.3 RESULTS 156
  - 5.3.1 Identification of isolate LD2 156
  - 5.3.2 Growth on and loss of carbazole 156
  - 5.3.3 Carbon and nitrogen limitation in isolate LD2 157
  - 5.3.4 Mineralization experiments 159
    - 5.3.4.1 Carbazole mineralization 159
    - 5.3.4.2 Mineralization of carbazole, glucose, and acetate 159
    - 5.3.4.3 Mineralization of labelled compounds in Norman Wells crude oil 161
  - 5.3.5 Metabolism of other aromatic compounds by isolate LD2 161
    - 5.3.5.1 Carbazole analogs 165
    - 5.3.5.2 Other aromatic compounds 169
  - 5.3.6 Growth on anthranilic acid and catechol 171
  - 5.3.7 Identification of carbazole metabolites 176
    - 5.3.7.1 BSA-treated LD2 culture extracts 176
    - 5.3.7.2 Diazomethane-treated LD2 culture extracts 177
      - 5.3.7.2.1 Metabolites with aniline-type structures 184
      - 5.3.7.2.2 Metabolites with indole-type structures 186
      - 5.3.7.2.3 Metabolites identified by NMR 193

5.3.7.2.4 Other metabolites 199
5.3.8 Metabolites detected from other carbazole-degrading cultures 199
5.3.9 Metabolites detected from LD2 mutants 201
5.4 DISCUSSION 203
5.5 LITERATURE CITED 212
6. OVERALL DISCUSSION, SUMMARY, AND SUGGESTIONS FOR FUTURE RESEARCH 218
LITERATURE CITED 224
APPENDIX A 226
MEDIA COMPOSITIONS 226
Liquid media for culturing of aerobic bacteria 226
Liquid media for culturing of iron-reducers 227
Liquid medium for methanogenic enrichments 229
Agar plates 230
SOURCES OF CHEMICALS USED 231
APPENDIX B 233
NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY 233
NMR Methods 233
NMR Data Interpretation 234
Compound **VI** 234
Compound **X** 235
APPENDIX C 239
PREPARATION OF FE(II) STANDARDS FOR FERROZINE ASSAY 239
LITERATURE CITED (Appendix) 240

LIST OF TABLES

TABLE PAGE

2.1 Molecular weights and aqueous solubilities of the most abundant compounds present in coal tar creosote....7

2.2 Structures and carcinogenicity ratings of carbazole and some PAHs found in creosote....10

2.3 Metabolites identified in the aerobic biodegradation of PAHs present in creosote....17

3.1 Percentage removal of selected creosote compounds in the DT Mixed and RMH Soil culture supernatant after incubation at 10°C or 28°C for 4 or 8 weeks....82

3.2 Percentage of label recovered as $^{14}CO_2$ in creosote- and oil-degrading cultures incubated with ^{14}C-*n*-hexadecane or ^{14}C-phenanthrene for 7, 14, or 28 days....85

4.1 Stoichiometry and standard free energies of mineralization reactions of substrates used in the Borden sand iron reduction studies....130

4.2 The percentage of Fe(III) reduced by Borden sand cultures based on predicted and actual amount of Fe(II) produced....132

5.1 Summary of pure and mixed cultures used in carbazole biodegradation experiments....146

5.2 Aromatic compounds tested as growth substrates for *Pseudomonas* sp. LD2....164

5.3 Loss of carbazole and its analogs in LD2 cultures incubated for 7 days....166

5.4 Metabolites detected in other carbazole-degrading cultures and LD2 mutants....202

B.1 ^{1}H and ^{13}C NMR data of compound X in $CDCl_3$ solution....237

LIST OF FIGURES

FIGURE **PAGE**

2.1 General reactions in the bacterial oxidation of PAHs................................15

2.2 Proposed pathway for dibenzofuran degradation by bacteria.......................21

2.3 Proposed pathway for the bacterial degradation of carbazole.......................24

3.1 GC chromatograms of acidified supernatant extracts of a sterile control and the SL culture incubated with Sidney coal tar for 1 month............69

3.2 Compounds identified by GC-MS in the acidified extract of sterile B+N medium containing OCT...72

3.3 A comparison of the chromatograms of the acidified supernatant extracts of a sterile control and the three DT cultures incubated with OCT for 3 weeks..73

3.4 A comparison of the chromatograms of the acidified supernatant extracts of a sterile control and the SL, ERN BIO, ESSO AG, and RMH Soil cultures incubated with OCT for 3 weeks..............................74

3.5 The average percent removal of 3- and 4-ringed PAHs in creosote by seven mixed creosote-degrading populations incubated with OCT for 4 weeks or 8 weeks...76

3.6 The average percent removal of carbazole and 3- and 4-ringed PAHs by four mixed creosote-degrading laboratory populations incubated for 4 weeks with OCT supplemented with carbazole and phenol..................78

3.7 The average percent biodegradation of carbazole and 3- and 4-ringed PAHs by four mixed creosote-degrading populations incubated for 8 weeks with OCT supplemented with carbazole and phenol.........................80

3.8 Mineralization of ^{14}C-labelled aromatic compounds added to OCT in the creosote-degrading DT Mixed culture...84

3.9 GC chromatograms showing the saturate fraction of PB crude oil in a sterile control and the different extents of removal of these compounds by four mixed cultures..87

3.10 Methane production from sewage sludge incubated under methanogenic conditions with various amounts (percent by volume) of water-soluble OCT compounds and whole OCT.................................89

4.1 Fe(II) production in Borden sand cultures (aerobic core #2) incubated with glucose, lactate, acetate, propionate, or butyrate and Fe(OH)$_3$.............116

4.2 Fe(II) production in Borden sand cultures (sampled from the anaerobic zone) incubated with $Fe(OH)_3$ and (a) non-aromatic compounds or (b) aromatic compounds....119

4.3 Fe(II) production in anaerobic Borden sand cultures incubated with glucose, acetate, lactate, or benzoate in the presence of $Fe(OH)_3$ or NTA....122

4.4 Fe(II) production in Borden sand cultures after successive transfers into medium containing glucose, acetate, lactate, or benzoate and $Fe(OH)_3$....123

4.5 Fe(II) production in Borden sand transfer cultures incubated with the water-soluble fraction of OCT and $Fe(OH)_3$....125

4.6 Fe(II) production in Borden sand transfer cultures, incubated with water-soluble creosote compounds and $Fe(OH)_3$....126

4.7 Fe(II) production in Borden sand incubated with medium, water-soluble creosote compounds, and $Fe(OH)_3$, NTA, or Fe(III)-NTA....127

5.1 Growth of isolate LD2 in liquid medium with carbazole as the sole source of carbon, nitrogen, and energy....158

5.2 Mineralization of ^{14}C-carbazole by isolate LD2 and by the mixed carbazole-degrading cultures CM, DC, and D5....160

5.3 Mineralization of (a) ^{14}C-carbazole and ^{14}C-glucose and (b) ^{14}C-carbazole and ^{14}C-acetate by isolate LD2 when these compounds were supplied as co-substrates in B-N medium....162

5.4 Structures of some aromatic compounds tested as growth substrates for isolate LD2....163

5.5 Viable cell counts of isolate LD2 incubated with carbazole or carbazole and one of dibenzothiophene, dibenzofuran, or fluorene....167

5.6 Mass spectra of analog transformation products detected by GC-MS when isolate LD2 was incubated with carbazole and dibenzothiophene or carbazole and fluorene....168

5.7 Mass spectra of dibenzofuran degradation products detected in culture supernatants when isolate LD2 was incubated with carbazole and dibenzofuran....170

5.8 Growth of isolate LD2 on anthranilic acid and the loss of this compound in B-N medium....172

5.9 Loss of carbazole and anthranilic acid when a mixture of these compounds was supplied and incubated with either carbazole-grown or anthranilic acid-grown LD2 cells....174

5.10 Loss of anthranilic acid by glucose-, anthranilic acid-, or carbazole-grown LD2 cells in the presence or absence of CAP....175

5.11 A portion of the total ion current chromatogram from the GC-MS analysis of a diazomethane-treated extract from a culture of isolate LD2 grown on carbazole....178

5.12 The mass spectra and proposed structures of compounds **I** and **III**....179

5.13 The mass spectra and proposed structures of compounds **V** and **VIII**....180

5.14 The mass spectra and proposed structures of compounds **IX** and **II**....181

5.15 The mass spectra and proposed structures of compounds **VI** and **X**....182

5.16 The mass spectra of compounds **IV** and **VII**....183

5.17 Growth of isolate LD2 on indole-3-acetic acid and the loss of this compound in B-N medium....187

5.18 Loss of indole-3-acetic acid and anthranilic acid from an LD2 culture when these compounds were added as a mixture in B-N medium....188

5.19 Structures of some compounds discussed in the text: (a) oxindole-3-propanoic acid; (b) the formation of compound **VI**; (c) kynurenine yellow; (d) dimethyl ether of 2'-aminobiphenyl-2,3-diol; (e) 1,9a-dihydroxy-1-hydrocarbazole....190

5.20 The mass spectra of the methyl esters of three synthesized oxindole derivatives showing the base peak at m/z 145 or 146....191

5.21 The mass spectra of the methyl esters of three indole derivatives showing the base peak at m/z 130....192

5.22 The proposed formation of compound **X** from 6-(2-aminophenyl)-2-hydroxy-6-oxo-hexa-2,4-dienoic acid....196

5.23 The mass spectrum and proposed structure of dimethylated compound **Xa** originating from 6-(2-aminophenyl)-2-hydroxy-6-oxo-hexa-2,4-dienoic acid....198

5.24 Summary of the metabolites identified from carbazole degradation by isolate LD2....200

5.25 Proposed pathways of carbazole degradation by isolate LD2 via 6-(2-aminophenyl)-2-hydroxy-6-oxo-hexa-2,4-dienoic acid (Pathway 1) or indole-3-acetic acid (Pathway 2)....209

B.1 The two-dimensional HMBC spectrum of compound **X** in $CDCl_3$ solution, one dimensional proton spectrum, and the APT spectrum....236

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BSA	*N,O*-bis(trimethylsilyl)acetamide
BT	Benzothiophene
CAP	Chloramphenicol
CFB	Canadian Forces Base
DBT	Dibenzothiophene
DMF	*N,N*-Dimethylformamide
DNAPL	Dense non-aqueous phase liquid
EPA	Environmental Protection Agency
Fe(III)	Ferric iron
Fe(II)	Ferrous iron
FID	Flame ionization detector
FTIR	Fourier transmission infrared (spectroscopy)
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HMW	High-molecular-weight
HP	Hewlett Packard
HPLC	High performance liquid chromatography
IARC	International Agency for Research on Cancer
ISPG	Institute for Sedimentary and Petroleum Geology
Km	Kanamycin
LMW	Low-molecular-weight
NMR	Nuclear magnetic resonance (spectroscopy)
NPL	National Priorities List

continued on next page...

LIST OF ABBREVIATIONS

NSO	Nitrogen-, sulfur-, oxygen-
NTA	Nitrilotriacetic acid
OCT	Ontario coal tar creosote
PAH	Polycyclic aromatic hydrocarbons
PB	Prudhoe Bay
PCA	Plate count agar
TLC	Thin-layer chromatography
TSB	Tryptic soy broth

1. INTRODUCTION

In 1991, a controlled field experiment was set up to examine the fate of coal tar creosote organics in groundwater. This experiment was conducted at a well-characterized shallow sand aquifer at Canadian Forces Base (CFB) Borden, located northwest of Toronto, Ontario, Canada. This study, initiated by Dr. J.F. Barker at the Waterloo Centre for Groundwater Research, University of Waterloo, Waterloo, Ontario, involved numerous researchers in Canada and Denmark and examined various aspects of groundwater contamination by creosote organics. My role in this project involved examining the biodegradation of creosote compounds by laboratory cultures under ideal conditions, determining iron-reducing activity in Borden aquifer sediments, and studying the metabolism of carbazole, a nitrogen heterocycle commonly found in creosote.

The following is a brief description of the Borden aquifer project. A literature review relating to the research presented in this thesis follows in Chapter 2.

1.1 Aims of the Borden project

A long term field study was undertaken to evaluate the mobility, persistance, and fate of organic contaminants typically introduced into groundwater from coal tar, creosote, and petroleum wastes. These processes were to be studied to gain an understanding of how contaminants migrate through groundwater and how this information could be used to make decisions about remediation or control of contaminated plumes. Another research objective was to develop and demonstrate *in situ* remediation technologies.

1.2 The Borden aquifer and emplacement of creosote

The site used for the Borden project experiments is an unconfined, shallow aquifer at CFB Borden. This aquifer is located about 350 m down-gradient from an old landfill site which was in operation from 1970-1976, and has been well-characterized hydrogeologically. The landfill-leachate contaminant plume and natural migration characteristics of this aquifer have been well documented (MacFarlane *et al.*, 1983). This site has been used for other hydrogeological studies examining the fate of chlorinated solvents and petroleum hydrocarbons (Barker *et al.*, 1987; Barbaro *et al.*, 1992).

The aquifer sand is composed of fine to medium grain and underlain by a thick silty, clayey deposit. The aquifer extends to a depth of 9 m and the landfill leachate is confined to the bottom 2-3 m. The movement of the groundwater is essentially horizontal, with very little mixing between the overlying recharge water and the leachate plume. The average temperature of the Borden aquifer is 12°C (Mackay *et al.*, 1986).

The Borden aquifer contains a vast network of multilevel piezometers which were installed for previous studies used to delineate the leachate plume and examine solute transport within the aquifer. These allow for groundwater to be sampled at various depths. Additional multilevel sampling wells were installed in and around the creosote sources emplaced in order to monitor the leaching of creosote chemicals.

Two sources of coal tar creosote were emplaced below the water table in the shallow Borden aquifer. One source and its plume is for remediation studies, the other serves as a natural control. The creosote sources were emplaced on August 28, 1991 and details of the installation have been described by King *et al.* (1994). The commercial creosote used was amended with carbazole, phenol, *m*-xylene, and *p*-cresol in order to increase the concentration of these compounds so that their fates could be monitored. In addition, NaCl was added as a tracer. Approximately 70 kg of creosote was emplaced in the two sources, and the relative concentrations of several individual creosote components have been published (Fowler *et al.*, 1994).

1.3 Field and laboratory studies

Field experiments focussed on sampling groundwater from all of the monitoring points to gain an initial "snapshot" of contaminant distributions as well as monthly sampling from numerous locations to obtain breakthrough data. Up to 3000 groundwater samples were obtained for each snapshot sampling (done every 6 months to a year since August, 1991). About 12 selected wells were monitored monthly to observe contaminant concentration changes over time. Source coring was also performed at various times to determine the concentrations of creosote compounds still present in the source. Dissolved oxygen and redox indicators (Fe^{2+}, NO_3^-, SO_4^{2-}, CH_4) have also been monitored. To date, most phenolic compounds have been completely leached from the source and are presumed to have been completely biodegraded in the groundwater. Polycyclic aromatic hydrocarbons and nitrogen, sulfur, and oxygen heterocycles are still leaching from the source (Fowler *et al.*, 1994).

Laboratory studies have included: the dissolution of creosote organics in batch tests to predict leaching from the field source, the biotransformation of creosote compounds under aerobic aquifer conditions in laboratory microcosms, and the influence of substrate interactions in aerobic biotransformation of creosote organics in dynamic column experiments. In addition, studies of aerobic creosote biodegradation, microbial iron reduction, and carbazole biodegradation were done and are described in this thesis.

Ongoing remedial studies include: the use of *in situ* chemical oxidation to remediate the emplaced source and dissolved organics, the examination of "remediated" groundwater for reduced toxicity, and evaluation of the novel "funnel-and-gate" method of treatment (Starr and Cherry, 1994).

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2. LITERATURE REVIEW

2.1 Creosote as a wood preservative and extent of creosote contamination

Coal tar creosote, pentachlorophenol, and CCA (copper chromium arsenate) are the primary chemicals used in the wood-preserving industry to treat wood products like railway ties, utility poles, bridge timbers, marine pilings, and other large-sized lumber (Mueller *et al.*, 1989; Environment Canada, 1993). In the United States, it has been estimated that there are 600 wood-preserving plants which collectively use over 4.5 X 10^7 kg of creosote each year (Mattraw and Franks, 1986). In Canada, there are 27 plants in operation collectively utilizing 2.1 X 10^7 kg of creosote as a wood preservative per year. Of the creosote used in Canada, 54% is used to treat railway ties, 37% is used for marine pilings, and the remaining 9% are used for bridge deckings, timbers, and utility poles (Environment Canada, 1993).

Creosote, a complex mixture of organic compounds, does not pose a significant threat to the environment or human health when properly used, however serious potential health risks can result when creosote is misused, accidentally spilled, or improperly disposed. Creosote contamination can be associated with surface soils, surface waters (treatment lagoons or holding ponds), and groundwaters beneath these sources (Mueller *et al.*, 1989). In Canada, estimates of the amount of creosote waste entering the environment are not available for many sites, although there is an estimated 2.56 X 10^5 kg of moderately to highly contaminated soil at 11 creosote-treating facilities, and there are at least 13 other potentially creosote-contaminated sites (Environment Canada, 1993). In the United States, close to 1400 wood treatment facilities exist (King *et al.*, 1994), and more than 40 sites are on the U.S. Environmental Protection Agency National Priorities list (Rosenfeld and Plumb, 1991). Many of the creosote-contaminated sites in both Canada and the United States are currently being studied to understand the occurrence, transport, and fate of creosote chemicals which contaminate soils and groundwaters.

2.2 Chemical composition of creosote

Creosote is a complex mixture of chemicals which results from the distillation of coal tar at 200-300°C. Generally, it is composed of 85% polycyclic aromatic hydrocarbons (PAHs), 10% phenolic compounds, and 5% nitrogen-, sulfur-, and oxygen-heterocycles (NSOs) (Mueller *et al.*, 1989), although the specific composition of an individual creosote can vary. Creosote can contain more than 200 different aromatic compounds, ranging from relatively water-soluble low molecular weight compounds such as phenols to water-insoluble, 6-ringed PAHs (Environment Canada, 1993). Creosote samples from numerous sources have been found to be similar in chemical composition (Novotny *et al.*, 1981; Rosenfeld and Plumb, 1991). Although detailed lists of the constituents present in various creosotes have been published (Novotny *et al.*, 1981; Pereira and Rostad, 1986; Rosenfeld and Plumb, 1991; Fowler *et al.*, 1994), a simplified list of the principle compounds present in the phenolic, PAH, and NSO fractions of a typical creosote was compiled from field data obtained from creosote-contaminated sites by Mueller *et al.* (1989) and is given in Table 2.1. Many of the compounds present in creosote are on the U.S. Environmental Protection Agency (EPA) list of priority pollutants due to their potential mutagenic, carcinogenic, or teratogenic properties (Rosenfeld and Plumb, 1991).

2.3 Health risks associated with compounds in creosote

2.3.1 Phenols

Phenols originating from industrial processes are of great concern to the environment because of their potential toxicity and recalcitrance (Londry and Fedorak, 1992). Waste-water effluents resulting from petroleum and coal conversion processes can contain up to gram quantities of phenol per litre (Fedorak and Hrudey, 1988) which can be

Table 2.1 Molecular weights and aqueous solubilities of the most abundant compounds present in coal tar creosote (after Mueller *et al.*, 1989). A "-" indicates solubility not found.

COMPOUND	MW (g/mol)	AQUEOUS SOLUBILITY (mg/L @ 25°C, unless noted)
Phenolics		
Phenol	94.1	82,000 (15°C)
o-Cresol	108.1	25,920
m-Cresol	108.1	23,500 (20°C)
p-Cresol	108.1	24,000 (40°C)
2,5-Dimethylphenol	122.2	3544
3,5-Dimethylphenol	122.2	4888
2,3-Dimethylphenol	122.2	4570
2,4-Dimethylphenol	122.2	6232
2,6-Dimethylphenol	122.2	6049
3,4-Dimethylphenol	122.2	4766
PAHs		
Naphthalene	128.2	31.7
1-Methylnaphthalene	142.2	28.5
2-Methylnaphthalene	142.2	25.4
Biphenyl	154.2	7.5
Acenaphthene	154.2	3.9
2,3-Dimethylnaphthalene	156.2	3.0
2,6-Dimethylnaphthalene	156.2	2.0
Fluorene	166.2	2.0
Phenanthrene	178.2	1.3
Anthracene	178.2	0.07
Fluoranthene	202.3	0.26
Pyrene	202.3	0.14
Benzo(a)fluorene	216.3	-
Benzo(b)fluorene	216.3	0.002
Benzophenanthrene	228.2	-
Chrysene	228.2	0.002
Benzo(a)pyrene	252.3	0.003
NSO Compounds		
Aniline	93.1	3400
Indole	117.1	3558
Quinoline	129.2	6718 (20°C)
Isoquinoline	129.2	4522 (20°C)
2-Methylquinoline	143.2	-
4-Methylquinoline	143.2	-
Carbazole	167.2	1 (20°C)
Benzothiophene	134.2	130 (20°C)
Dibenzothiophene	184.3	2
Dibenzofuran	168.2	10

toxic to aquatic biota and degrade the quality of water to which the waste effluents from industry are being discharged. The toxicity of phenols has been studied on selected microbes, algae, invertebrates, and vertebrates. Depending on the organism, acute toxicity can vary from 6.5 to 1840 mg/L phenol, and studies have shown that higher organisms like fish are most susceptible to the toxic effects of phenols (Buikema and McGinniss, 1979). The phenols which comprise about 10% of coal tar creosote are generally the most abundant compounds present in the groundwaters of creosote-contaminated sites because of their relatively high water solubilites (Mueller *et al.*, 1989). Because of their mobility in groundwater, phenols are often detected at the leading edge of a contaminant plume which has implications for contaminant containment and remediation.

2.3.2 PAHs and NSO compounds

The PAHs are a group of hydrophobic and highly lipophilic compounds that are ubiquitous as pollutants in the environments of industrialized areas of the world. The concentration of PAHs can vary widely, ranging from 5 ng/g soil in an undeveloped area to 1.79 mg/g soil at an industrial plant (Cerniglia, 1992). Generally, the toxicity of a PAH increases with increasing molecular size. For example, naphthalene, a two-ringed PAH, has not been found to be genotoxic, whereas benzo(a)pyrene, a five-ringed PAH, has been found to be carcinogenic and teratogenic in laboratory animals (IARC, 1983; Cerniglia, 1992). Several PAHs have been found to bioaccumulate in aquatic biota and adverse toxicological effects have resulted in these organisms (Environment Canada, 1993).

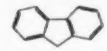
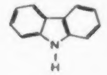
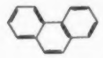
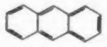
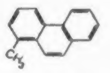
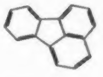
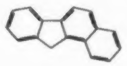
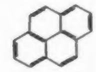
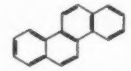
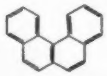
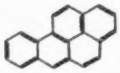
In addition, NSO compounds have also been found to be potentially harmful to the environment and to human health (Schultz, 1983; West *et al.*, 1986). S- and N-heterocycles are thought to be more toxic than their analogous PAHs. For example, a neutral subfraction of coal-derived liquids containing N-heterocycles was found to have twice the mutagenic activity of a neutral PAH subfraction (Santodonato and Howard, 1981). While some PAHs and NSO compounds are toxic if they are inhaled, ingested, or exposed to skin, not all have been found to be mutagenic or carcinogenic. The structures and carcinogenicity ratings of carbazole and selected PAHs present in creosote have been compiled in Table 2.2, based on data obtained by the International Agency for Research on Cancer (IARC, 1983). Studies with humans and animals, as well as short-term tests (such as the Ames test) were used to evaluate carcinogenicity. In many cases, there is a lack of data available from human studies, so if a chemical is found to be carcinogenic in laboratory animals, it is regarded as carcinogenic in humans. Because of the toxic effects of many creosote chemicals, there is great interest in studying the fate of these chemicals entering the environment.

2.4 Fate of creosote chemicals in the environment

Creosote can enter the environment via losses during production, transport, storage, and accidental spillage, or by improper waste disposal at creosote manufacturing or treatment facilities, resulting in a deterioration of ground- and surface-water quality (Pereira and Rostad, 1986). In many cases, creosote chemicals enter the groundwater of an aquifer which often serves as the primary source of drinking water for a community (Mattraw and Franks, 1986). In the United States, groundwater supplies about 25% of fresh water used for drinking, industrial, and agricultural purposes and accounts for almost 50% of all drinking water supplies (Bouwer, 1992; Liu *et al.*, 1992). Furthermore, approximately 38% of municipalities in Canada rely partially or solely on groundwater for their water supply. The heavy human reliance on groundwater necessitates the protection of groundwater sources from chemical contamination.

The fate of organic contaminant(s) entering the ground depends on both the characteristics of the soil and groundwater at a contaminated site and the properties of the contaminating compounds. Soil characteristics such as texture, density, pore size, ion exchange capacity, organic matter content, pH, and water holding capacity as well as hydrogeological factors like porosity, permeability, and groundwater flow patterns and

Table 2.2 Structures and carcinogenicity ratings of carbazole and some PAHs present in creosote (from IARC, 1983).

COMPOUND	STRUCTURE	CARCINOGENICITY RATING*	
		Animals	Short Term
Fluorene		+/-	+/-
Carbazole		(+)	+/-
Phenanthrene		+/-	(+)
Anthracene		-	-
1-Methylphenanthrene		+/-	+
Fluoranthene		-	+/-
Benzo(a)fluorene		+/-	+/-
Pyrene		-	(+)
Chrysene		(+)	(+)
Benzo(c)phenanthrene		+/-	+/-
Benzo(a)pyrene		+	+

* + = sufficient evidence - causal relationship shown in several studies
(+) = limited evidence - suggested carcinogenic effect, but limited studies
+/- = inadequate evidence - insufficient or conflicting evidence
- = no evidence - several studies show no evidence

characteristics influence the migration of pollutant compounds through the subsurface (Sims *et al.*, 1990; Bouwer, 1992). Chemical properties such as structure, water-solubility, reactivity, and concentration will influence the migration of contaminants through different zones of the subsurface environment (Sims *et al.*, 1990). Coal tar creosote, for example, contains compounds of various water solubilities which will determine the rate of migration of these compounds through groundwater. The microbial communities present in soils and groundwaters and the availability of terminal electron acceptors (for example, oxygen, nitrate, ferric iron, or sulfate) will also determine the fate of contaminants.

When organic compounds are spilled on the land, they first migrate through the unsaturated zone (vadose zone) where their movement can be prevented by processes such as chemical precipitation and degradation, adsorption to soil particles, volatilization, or biological transformation (Pereira and Rostad, 1986). When a soil is heavily contaminated, many diverse compounds migrate further downward past the water table and enter the groundwater, or the saturated zone. In the saturated zone, the fate of contaminants is also influenced by various physical, chemical, and biological processes including advection, dilution, precipitation, sorption, oxidation-reduction reactions, and biological assimilation and degradation (Pereira and Rostad, 1986; Barker *et al.*, 1987).

Because creosote is a dense (heavier than water) non-aqueous phase liquid (DNAPL), it tends to migrate through the vadose zone and penetrate to depths below the water table. The more water-soluble compounds present in creosote, such as phenols and low-molecular-weight (LMW) PAHs and NSO compounds, dissolve relatively quickly in the groundwater, and can thus be transported downgradient away from the spill source along with groundwater flow (Arvin *et al.*, 1989; Environment Canada, 1993). The denser, less water-soluble compounds in creosote often travel downwards until they encounter an impervious layer in the subsurface. As the denser hydrocarbons and heterocycles percolate downwards through the saturated zone, some compounds will adsorb to subsurface material and some compounds will slowly dissolve in the flowing groundwater and also be transported downgradient from the contaminant source. For a given site where creosote contamination of the groundwater has occurred, the more water-soluble compounds such as phenol, naphthalene, and quinoline have been detected at points farthest away from the contaminant source whereas higher-molecular-weight (HMW) compounds such as phenanthrene and anthracene remain closer to the contaminant source (Erlich *et al.*, 1982; Godsey *et al.*, 1992; Fowler *et al.*, 1994). The occurrence and distribution of creosote contaminants in the subsurface can be complex due to interactions between the chemicals and the heterogeneous nature of the subsurface environment (Barker *et al.*, 1994).

2.5 Biodegradation of creosote compounds

Both non-biological and biological processes influence the fate of creosote chemicals in the subsurface environment. Although losses of compounds may occur via abiotic processes such as volatilization, leaching, and photodegradation (Mueller *et al.*, 1989), it has become increasingly evident that the biological degradation of creosote compounds contributes significantly to the losses of these compounds at contaminated sites (Mueller *et al.*, 1989; Sims *et al.*, 1990; Madsen, 1991). Studies which have examined the microbial ecology of aquifer environments have shown that a wide variety of aerobic and anaerobic microorganisms are present (for review, see Madsen and Ghiorse, 1993) and bacterial populations have been isolated from many sources, including creosote-contaminated sites, which are capable of degrading aromatic compounds under various redox conditions (Evans and Fuchs, 1988). Dozens of studies have shown that many aromatic compounds present in creosote are biodegraded naturally in contaminated groundwater or as a result of various bioremediation strategies applied to contaminated sites.

The biodegradation of aromatic compounds has been studied intensively in the laboratory and as a result, much is known about the catabolism of these compounds. Excellent reviews have recently been written about the biodegradation of aromatic compounds under aerobic (Cerniglia, 1984; Gibson and Subramanian, 1984; Cerniglia, 1992) and anaerobic (Berry *et al.*, 1987a; Evans and Fuchs, 1988; Londry and Fedorak, 1992) conditions. The following sections discuss the biodegradation of the major components of coal tar creosote under aerobic and anaerobic conditions.

2.6 Aerobic degradation of creosote compounds

2.6.1 Phenols

The biodegradation of phenols, including methyl and dimethylphenols, under aerobic conditions has been thoroughly studied and reviewed. Gibson and Subramanian (1984) outline the metabolic pathways for phenol and *o*-, *m*-, *p*-cresols in bacteria, and the work which has been done to elucidate the genetic regulation of these pathways. The aerobic degradation of phenols proceeds through a catechol intermediate which then serves as a substrate for catechol 1, 2- or 2, 3-dioxygenases resulting in *ortho* or *meta* cleavage, respectively. The mechanism of degradation of dimethylphenols has been found to vary depending on the pattern of methyl substitution (Bayly and Barbour, 1984; Ewers *et al.*, 1989). In the aerobic degradation of phenols, citric acid cycle intermediates like acetic acid, malic acid, and fumaric acid are ultimately produced, and thus the compounds are eventually broken down to CO_2 and H_2O (Gibson and Subramanian, 1984).

2.6.2 Polycyclic aromatic hydrocarbons

The biodegradation or biotransformation of PAHs has been found to occur by mixed and pure populations of bacteria, fungi, and algae (Cerniglia, 1992). A fairly exhaustive list of the various species of microorganisms that can biotransform or mineralize

PAHs under aerobic conditions has been presented by Cerniglia (1992). Some genera of bacteria able to oxidize PAHs include *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Beijerinckia*, *Flavobacterium*, *Mycobacterium*, *Pseudomonas*, and *Rhodococcus*. In a review on the biodegradation of PAHs, Cerniglia (1992) outlines many of the general principles which apply to the bacterial metabolism of PAHs under aerobic conditions. These general principles (modified somewhat) are summarized below:

- LMW compounds are more rapidly biodegraded than HMW compounds.
- The biochemical pathways for PAHs containing three or less aromatic rings have been elucidated, whereas less is known about the biodegradation of PAHs containing four or more aromatic rings.
- Compounds containing more than four aromatic rings are generally resistant to microbial attack.
- The presence of molecular oxygen is necessary for the initiation of enzymatic attack of PAHs.
- During initial oxidation of a PAH, bacteria stereoselectively incorporate both atoms of molecular oxygen using a dioxygenase enzyme to form a *cis*-dihydrodiol.
- The catabolic genes necessary for the degradation of PAHs are often located on plasmids.

The general reactions involved in the oxidation of PAHs are shown in Fig. 2.1 (after Cerniglia, 1984; Gibson, 1993). The bacterial metabolism of naphthalene is well understood and much of what is known about the metabolism of PAHs is based on studies with this compound. The dioxygenase that initially oxidizes naphthalene is a three-component enzyme system consisting of (1) a reductase which transfers electrons from NADH or NADPH to (2) a ferrodoxin which in turn reduces (3) the terminal iron-sulfur protein that catalyzes the stereoselective oxidation of naphthalene to *cis*-1,2-dihydroxy-1,2-

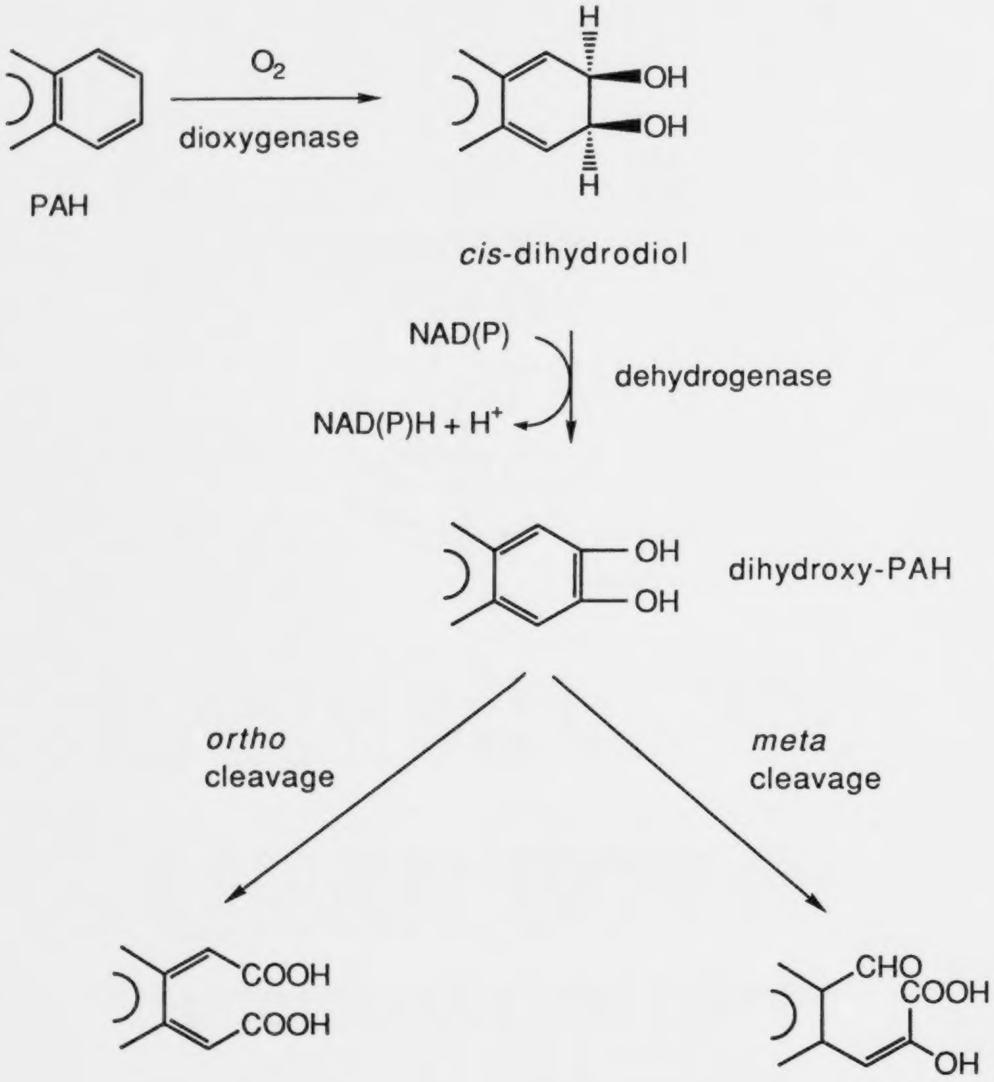

PAH
O_2
dioxygenase
OH
OH
H
H
cis-dihydrodiol
NAD(P)
NAD(P)H + H⁺
dehydrogenase
OH
OH
dihydroxy-PAH
ortho
cleavage
meta
cleavage
COOH
COOH
CHO
COOH
OH

Figure 2.1 General reactions in the bacterial oxidation of PAHs.

dihydronaphthalene. The *cis*-dihydrodiol is then rearomatized via the action of a dehydrogenase to yield 1,2-dihydroxynaphthalene (Cerniglia, 1984). Depending on the compound and the bacterium, this catechol-type structure can then be further oxidized via the *meta* pathway in which the bond between one of the carbon atoms containing a hydroxyl group and an adjacent carbon atom is cleaved to yield a hydroxymuconic semialdehyde structure (as is the case for naphthalene) or via the *ortho* pathway, in which the bond between the two carbon atoms containing the hydroxyl groups is cleaved to yield a *cis, cis*-muconic acid structure. Salicylic acid is a commonly produced naphthalene metabolite, although catechol and gentisic acid have both been found as further breakdown products of salicylic acid in different bacteria. Divergent metabolic pathways have also been observed in phenanthrene biodegradation by bacteria (Gibson and Subramanian, 1984).

A list of many PAHs found in coal tar creosote which have been shown to be amenable to bacterial degradation and their reported metabolites are given in Table 2.3. While many 2- and 3-ringed PAHs can be broken down into easily degradable aromatic acids or catechol, and ultimately into simple, innocuous compounds which can enter into the citric acid cycle, the only breakdown products identified to date for HMW compounds like benzo(a) pyrene have been *cis*-dihydrodiols. For some compounds, although breakdown products have not been identified, they have been shown to be degradable by bacteria. For example, *Rhodococcus* sp. RW1, a pyrene-degrading bacterium, was also found to use chrysene as a carbon and energy source for growth, although chrysene metabolites were not reported (Walter *et al.*, 1991).

Table 2.3 Metabolites identified in the aerobic biodegradation of PAHs present in creosote.

COMPOUND	METABOLITES	REFERENCE(S)
Acenaphthene	1-acenaphthenol 1-acenaphthenone 1,2-acenaphthenediol 1-hydroxy-2-ketoacenaphthene acenaphthenequinone	Schocken & Gibson, 1984
Anthracene	*cis*-anthracenedihydrodiol 1,2-dihydroxyanthracene 4-(2'-hydroxynaphth-3-yl)-2-oxo-4-hydroxybutyric acid 2-hydroxy-3-naphthaldehyde 2-hydroxy-3-naphthoic acid 2,3-dihydroxynaphthalene salicylic acid catechol	Gibson & Subramanian, 1984 Atlas, 1984
Benzo(a)pyrene	*cis*-9,10-dihydrodiol *cis*-7,8-dihydrodiol	Gibson & Subramanian, 1984
Biphenyl	*cis*-2,3-dihydro-2,3-dihydroxybiphenyl 2,3-dihydroxybiphenyl 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid 2-hydroxy-6-oxo-6-phenylhexa-2-enoic acid 2,6-dioxo-6-phenylhexanoic acid γ-benzoylbutyraldehyde γ-benzoylbutyric acid 2-oxopent-4-enoic acid benzoic acid catechol	Gibson & Subramanian, 1984 Takase *et al.* 1986 Omori *et al.* 1988
Fluoranthene	7-hydroxyacenaphthylene 7-acenaphthenone 3-hydroxymethyl-4,5-benzocoumarin	Weissenfels *et al.* 1991
Fluorene	9-fluorenol 9-fluorenone 1,1a-dihydroxy-1-hydro-9-fluorenone 3-(2'-carboxyphenyl)catechol 2-hydroxy-6-(2-carboxyphenyl)-6-oxo-2,4-hexadienoic acid phthalic acid 2-oxo-4-pentenoate	Trenz *et al.* 1994 Grifoll *et al.* 1994

Table 2.3 continued on next page...

COMPOUND	METABOLITES	REFERENCE(S)
1-Methylnaphthalene	3-methylsalicylic acid 3-methylcatechol	Dean-Raymond & Bartha, 1978
2-Methylnaphthalene	4-methylcatechol	Williams *et al*. 1975
Naphthalene	*cis*-naphthalenedihydrodiol 1,2-dihydroxynaphthalene 1,2-naphthoquinone 2-hydroxy-4-(2'-oxo-3,5-cyclohexadienyl)-buta-2,4-dienoate *cis-o*-hydroxybenzylidenepyruvate 2-hydroxychromene-2-carboxylate *trans-o*-hydroxybenzylidenepyruvate salicylaldehyde salicylic acid	Eaton & Chapman, 1992
Phenanthrene	*cis*-phenanthrenedihydrodiol 3,4-dihydroxyphenanthrene *cis*-4-(1-hydroxynaphth-2-yl)-2-oxobut-3-enoic acid 1-hydroxy-2-naphthaldehyde 1-hydroxy-2-naphthoic acid 2-carboxy-benzaldehyde *o*-phthalic acid protocatechuic acid	Gibson & Subramanian, 1984
	1,2-dihydroxynaphthalene *cis-o*-hydroxybenzylpyruvic acid salicylaldehyde salicylic acid catechol	Atlas, 1984
Pyrene	1,2-dihydroxypyrene pyrenol 4-hydroxyperinaphthenone 4-phenanthroic acid phthalic acid cinnamic acid	Heitkamp *et al*. 1988
	4,5-dihydroxypyrene 2-hydroxy-2-(phenanthren-5-one-4-enyl)-acetic acid	Walter *et al*. 1991

2.6.3 Nitrogen-, sulfur-, and oxygen-heterocycles

2.6.3.1 Sulfur-heterocycles

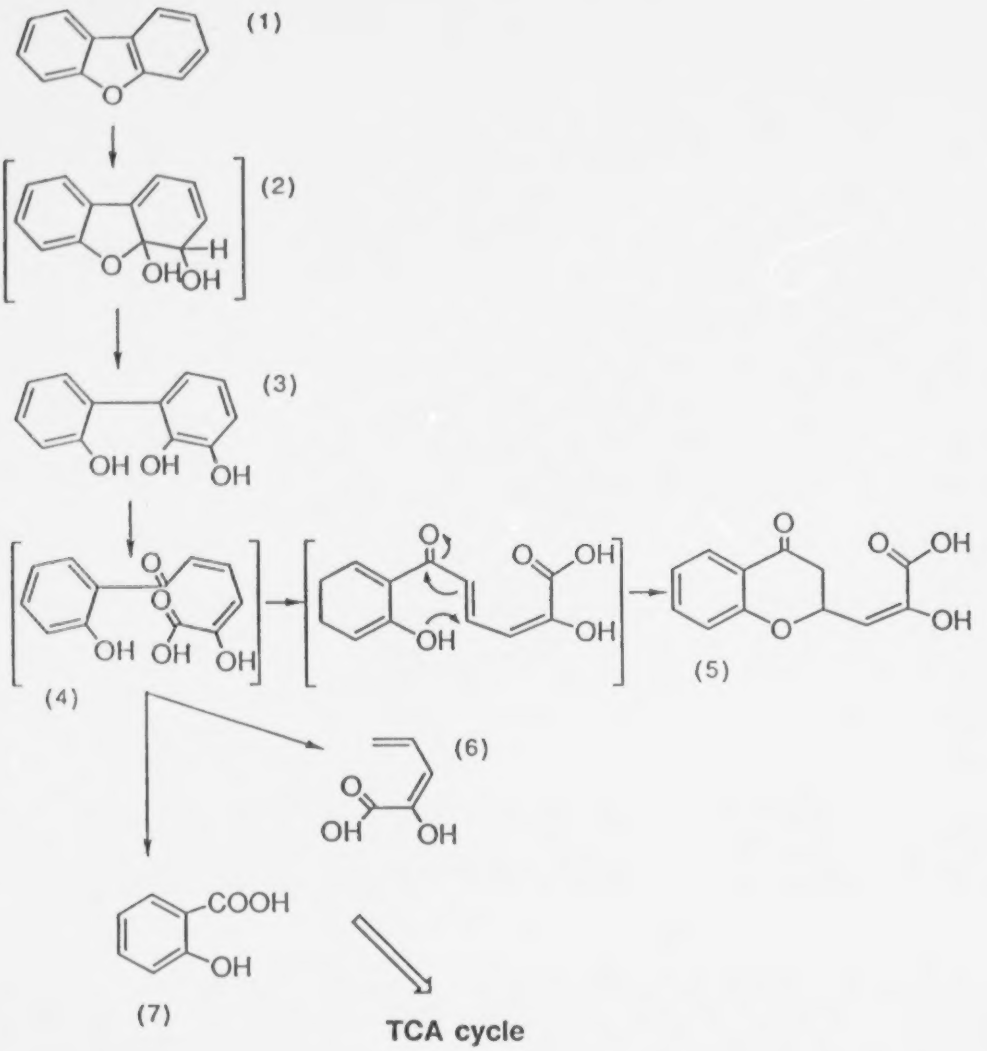
In comparison with the amount of information that is known on the bacterial degradation of PAHs, relatively little is known about the degradation of heterocyclic compounds. Of the sulfur-heterocycles, benzothiophene (BT) and dibenzothiophene (DBT) have been well studied, and many of the methylated derivatives of BT and DBT can be biotransformed by bacteria (Fedorak and Grbic´-Galic´, 1991; Saftic´ *et al.*, 1992; Saftic´ *et al.*, 1993; Kropp *et al.*, 1994). These biotransformations generally produced sulfoxides or sulfones, although oxidation of methyl groups to carboxylic acids has been observed for some methyl-substituted BTs (Kropp *et al.*, 1994). Species of *Pseudomonas*, *Acinetobacter*, *Beijerinckia*, and *Rhizobium* have been shown to oxidize DBT to 3-hydroxy-2-formylbenzothiophene, leaving the thiophene ring intact (Krawiec, 1990). Bacteria have also been isolated which are able to degrade dibenzothiophene by selectively oxidizing the sulfur atom to a sulfoxide and a sulfone which are intermediates in the '4S' pathway. In this mechanism of DBT degradation, sulfur is released as sulfate, and metabolites such as biphenyl, 2,2'-dihydroxybiphenyl, and 2-hydroxybiphenyl are produced (Krawiec, 1990; Omori *et al.*, 1992).

2.6.3.2 Oxygen-heterocycles

Dibenzofuran is the predominant oxygen heterocycle found in creosote and many products of its degradation by bacteria have recently been identified. In ground-breaking work, Engesser *et al.* (1989) found that two dibenzofuran-degrading *Brevibacterium* strains transformed the PAH fluorene into 1,10-dihydroxy-1,10-dihydrofluoren-9-one, suggesting the presence of a novel dioxygenase which attacked polynuclear aromatic systems in an unusual angular position. The initial oxidation of dibenzofuran was proposed to occur in the same manner. Other studies by this (Strubel *et al.*, 1991) and another group (Fortnagel *et al.*, 1990) confirmed this mode of angular dioxygenation of dibenzofuran with the identification of 2,2',3-trihydroxybiphenyl as an intermediate (see Fig. 2.2, for a dibenzofuran degradation pathway). Angular dioxygenation of dibenzofuran yields an unstable hemiacetal which decays spontaneously and is subsequently rearomatized by dehydration to the trihydroxybiphenyl (Engesser *et al.*, 1989). *meta* Cleavage of 2,2',3-trihydroxybiphenyl yielded 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)-hexa-2,4-dienoic acid (HOHPDA) and subsequently salicylic acid and 2-oxo-4-pentanoate, in a manner analogous to biphenyl metabolism (Fortnagel *et al.*, 1990; Strubel *et al.*, 1991). HOHPDA, an unstable compound, was not actually isolated; this compound was found to undergo spontaneous intramolecular Michael addition to form 3-(chroman-4-on-2-yl)pyruvic acid, which was characterized by nuclear magnetic resonance spectroscopy (Strubel *et al.*, 1991). Fortnagel *et al.* (1990) identified numerous dibenzofuran metabolites with 2-substituted chroman-4-one type structures, presumably cyclization products of unstable, enzymatically-produced dibenzofuran metabolites. Dibenzofuran 4,4a-dioxygenase, which catalyzed angular dioxygenation of dibenzofuran in *Sphingomonas* sp. strain RW1, was found to be a three-component enzyme system and two isofunctional hydrolases involved in the dibenzofuran degradation by this bacterium have been purified (Bunz and Cook, 1993; Bunz *et al.*, 1993).

2.6.3.3 Nitrogen-heterocycles

Of the nitrogen heterocycles found in coal tar creosote, the biodegradation of the 2-ringed quinoline and its methylated derivatives has been the most frequently studied. Bennett *et al.* (1985) isolated four *Pseudomonas* sp. from creosote-contaminated soil which oxidized quinoline to 2-hydroxyquinoline, confirming previous work (Grant and Al-Najjar, 1976). Studies with other *Pseudomonas* spp. and *Comamonas acidovorans* DSM 6426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O
O
OH
OH
(5)
(6)
O
OH
OH
COOH
OH
(7)
TCA cycle

(1) dibenzofuran
(2) 4,4a-dihydro-4,4a-dihydroxydibenzofuran
(3) 2,2',3-trihydroxybiphenyl
(4) 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4-hexadienoic acid
(5) 3-(chroman-4-on-2-yl)pyruvic acid
(6) 2-hydroxy-4-pentenoate
(7) salicylic acid

Figure 2.2 Proposed pathway for dibenzofuran degradation by bacteria (after Strubel *et al.*, 1991).

showed that 2-hydroxyquinoline was further degraded to 2,8-dihydroxyquinoline, 8-hydroxycoumarin, and 2,3-dihydroxyphenyl-3-propionic acid after attack of the pyridine ring (Shukla, 1986; Miethling *et al.*, 1993). *meta* Cleavage of the latter product results in compounds which enter the citric acid cycle.

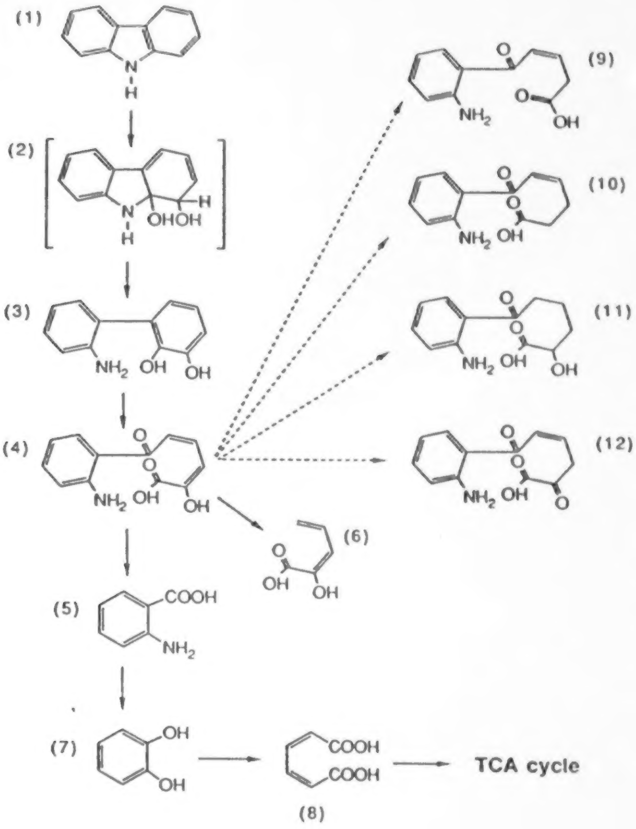
After the formation of 2-hydroxyquinoline, species of *Nocardia*, *Rhodococcus*, and *Moraxella* attacked the benzylic ring of quinoline yielding 6- and 5,6-dihyroxy-2-oxo-1,2-dihydroquinoline. Further cleavage produced 5-hydroxy-6-(3-carboxy-3-oxopropenyl)-1-H-2-pyridone which could be channeled to the citric acid cycle (Miethling *et al.*, 1993). *Pseudomonas aeruginosa* strain QP, a quinoline-degrader isolated by Aislabie *et al.* (1990) harbored three plasmids which contained catabolic genes for quinoline degradation. 2-Methylquinoline was also degraded by a *Pseudomonas* strain isolated by this group and a pathway for the degradation of this compound by *Alcaligenes* and *Arthrobacter* spp. was reported by Dembek *et al.* (1989). Gram-negative bacteria from deep subsurface sediments have also been found to mineralize quinoline (Brockman *et al.*, 1989).

Indole, a N-containing compound commonly found in creosote-contaminated groundwaters (Fowler *et al.*, 1994), is also biodegradable under aerobic conditions. *Alcaligenes* sp. strain In3 was shown to degrade indole to indoxyl, isatin, anthranilic acid and gentisic acid in sequential order (Claus and Kutzner, 1983). Anthranilic acid was also a product of indole degradation by a Gram-positive bacterium isolated from soil (Fujioka and Wada, 1968), although its formation proceeded via 2,3-dihydroxyindole.

When work on this project began in 1991, very little was known about the aerobic degradation of carbazole, a 3-ringed N-heterocycle. Studies conducted in the early 1970s showed that ovalic acid, L-tryptophan, and 3-(3-oxo-2-indolinyl)propionic acid accumulated in culture medium when *Pseudomonas ovalis* UB-1701 was incubated in the presence of carbazole (Yamada *et al.*, 1974a, b, and c). Finnerty *et al.* (1983) studied carbazole as a model compound for the biological denitrogenation of fossil fuels. In this work, four bacteria were isolated which could degrade carbazole, two of which could use carbazole as a sole source of carbon, nitrogen, and energy. Although degradation products were observed by thin-layer chromatography, no metabolite identifications were made. Grosser *et al.* (1991) isolated a carbazole-degrading *Xanthomonas* sp. from contaminated soil. When this bacterium was reintroduced into contaminated soil, enhanced carbazole mineralization was observed.

Two *Pseudomonas* strains, CA06 and CA10 were isolated by Ouchiyama *et al.* (1993) that could utilize carbazole as a sole source of carbon and nitrogen for growth. These authors positively identified anthranilic acid and catechol as carbazole metabolites, and tentatively identified numerous others based on gas chromatography-low resolution mass spectrometry analyses. On the basis of these results, a pathway was proposed for the degradation of carbazole by these isolates and is shown in Fig. 2.3. The proposed initial oxidation product, 1,9a-dihydroxy-1-hydrocarbazole, would result from dioxygenation in the angular position as has been observed for fluorene and dibenzofuran metabolism (Grifoll *et al.*, 1994; Engesser *et al.*, 1989; Strubel *et al.*, 1991). By analogy to the pathway for biphenyl metabolism, 2'-amino-2,3-dihydroxybiphenyl, the subsequent degradation product, was proposed to undergo *meta* cleavage to 2-hydroxy-6-oxo-(2'-aminophenyl)hexa-2,4-dienoic acid which in turn was cleaved to form anthranilic acid and 2-hydroxy-4-pentenoate. Transposon mutants of strain CA10 were later made and genes for the enzymes involved in the degradation of carbazole were isolated and analyzed (Omori, 1995).

Two other studies on carbazole degradation with *Pseudomonas stutzeri* strain SR250 (Resnick and Gibson, 1993) and another *P. stutzeri* strain (Hisatsuka and Sato, 1994) also showed anthranilic acid as a carbazole metabolite. Mutant strains of *P. stutzeri* SR250 incubated with carbazole also accumulated 1-hydroxycarbazole which was thought to have resulted from the rearomatization of an unstable "angular 1,9a-diol" (Resnick and Gibson, 1993). Resnick *et al.* (1993) observed 3-hydroxycarbazole, presumed to result from the dehydration of an unstable *cis*-dihydrodiol, an oxidation product of carbazole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COOH
TCA cycle
(9)
O
O
NH₂ OH
(10)
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O
NH₂ OH
(11)
O
O
NH₂ OH OH
(12)
O
O
NH₂ OH O

(1) carbazole
(2) 1,9a-dihydroxy-1-hydrocarbazole
(3) 2'-aminobiphenyl-2,3-diol
(4) 2-hydroxy-6-oxo-6-(2'-aminophenyl)hexa-2,4-dienoic acid
(5) anthranilic acid
(6) 2-hydroxy-4-pentenoate
(7) catechol
(8) *cis, cis*-muconic acid
(9) 5-oxo-5-(2'-aminophenyl)penta-3-enoic acid
(10) 6-oxo-6-(2'-aminophenyl)hexa-4-enoic acid
(11) 2-hydroxy-6-oxo-6-(2'-aminophenyl)hexanoic acid
(12) 2,6-dioxo-6-(2'-aminophenyl)hexa-4-enoic acid

Figure 2.3 Proposed pathway for the bacterial degradation of carbazole (after Ouchiyama *et al.*, 1993).

by strains with naphthalene 1,2-dioxygenase and biphenyl 2,3-dioxygenase activity.

Completely different carbazole metabolites were proposed by Grosser *et al.* (1994) when a *Xanthomonas* sp. was incubated with carbazole as a sole carbon source, including indoleacetic acid, indolepropionaldehyde, and *cis*-indoleacrylic acid. Studies by Agarwal *et al.* (1995) showed that benzoate was produced when *P. aeruginosa* strain SAG-S was incubated with carbazole as a sole carbon source. The carbazole catabolic genes for this bacterium were located on a 150 kb plasmid.

2.7 Anaerobic degradation of creosote compounds

When creosote-contaminants reach the subsurface environment and a contaminant plume develops, the oxygen present in the groundwater is rapidly consumed by aerobic microorganisms, leaving anaerobic conditions to prevail. Within a contaminant plume, different redox zones will develop according to the oxidants (terminal electron acceptors) present in the subsurface which are available for use by microorganisms to degrade the contaminant compounds (Christensen *et al.*, 1994). Geochemical evidence suggests that nitrate, Fe(III), Mn(IV), sulfate, and CO_2 are the primary terminal electron acceptors for organic matter degradation in anaerobic environments (Lovley and Phillips, 1988). A subsurface microbial community will utilize the terminal electron acceptor available which will yield the greatest amount of energy (Londry and Fedorak, 1992). For example, if oxygen is available, it will be utilized first as a terminal electron acceptor, followed by nitrate. Oxidized forms of iron and manganese will then be used as terminal electron acceptors if available, followed by the use of sulfate. Finally, in the absence of other electron acceptors, methane may be produced by fermentation of specific compounds or by carbon dioxide (or bicarbonate) reduction (Londry and Fedorak, 1992; Christensen *et al.*, 1994). That portion of the population which obtains the greatest amount of energy from a particular terminal electron acceptor will outcompete others. For example, iron-reducing bacteria have been found to outcompete sulfate-reducers and methanogens (Lovley and Phillips, 1988; Chapelle and Lovley, 1992).

The development of different redox zones within a shallow aquifer contaminated with organic compounds generally occurs, although it can be difficult to accurately delineate the distribution of redox zones because of variations within microenvironments and short-term temporal and spacial variations of terminal electron-accepting processes (Christensen *et al.*, 1994; Vroblesky and Chapelle, 1994). Despite this, various redox processes have been identified in numerous aquifers (Christensen *et al.*, 1994) and recently, dissolved hydrogen concentrations in groundwater have been used to determine the distribution of redox zones (Lovley and Goodwin, 1988; Vroblesky and Chapelle, 1994; Lovley *et al.*, 1994a). The following equations show the amount of energy (expressed by Gibbs free-energy changes at pH 7) obtained in the degradation of a model organic compound, CH_2O, in the presence of different terminal electron acceptors (after Christensen *et al.*, 1994):

Aerobic:

$CH_2O + O_2 \rightarrow CO_2 + H_2O$ $\Delta G^{\circ\prime} = -120$ kcal/mol (-502 kJ/mol)

Nitrate reduction:

$5CH_2O + 4NO_3^- + 4H^+ \rightarrow 5CO_2 + 2N_2 + 7H_2O$ $\Delta G^{\circ\prime} = -114$ kcal/mol (-477 kJ/mol)

Manganese reduction:

$CH_2O + 2MnO_2 + 4H^+ \rightarrow CO_2 + 2Mn^{2+} + 3H_2O$ $\Delta G^{\circ\prime} = -81$ kcal/mol (-339 kJ/mol)

Iron reduction:

$CH_2O + 4Fe(OH)_3 + 8H^+ \rightarrow CO_2 + 4Fe^{2+} + 11H_2O$ $\Delta G^{\circ\prime} = -28$ kcal/mol (-117 kJ/mol)

Sulfate reduction:

$2CH_2O + SO_4^{2-} + H^+ \rightarrow 2CO_2 + HS^- + 2H_2O$ $\Delta G^{\circ\prime} = -25$ kcal/mol (-105 kJ/mol)

Methanogenesis:

$2CH_2O \rightarrow CH_3COOH \rightarrow CH_4 + CO_2$ $\Delta G^{\circ\prime} = -22$ kcal/mol (-92 kJ/mol)

In an aquifer, oxygen, nitrate, and sulfate may be dissolved in the groundwater, whereas iron and manganese are associated with the aquifer solids (Christensen *et al.*, 1994). Iron is expected to be the major contributor to the oxidation capacity in most aerobic aquifers (Christensen *et al.*, 1994) and ferric iron is often the most abundant terminal electron acceptor for microbial metabolism when soils and sediments become anaerobic (Lovley, 1991). While the biodegradation of organic compounds under methanogenic, nitrate-reducing, and sulfate-reducing conditions has been studied for many years (for reviews see Berry *et al.*, 1987a; Evans and Fuchs, 1988), the importance of Fe(III) as a terminal electron acceptor in the subsurface environment has only been realized in the last ten years or so. Iron reduction as an anaerobic process will be discussed in more detail in Section 2.8. Although not discussed here, it should be noted that dissimilatory Mn(IV) reduction has also received recent attention as an important anaerobic process and has been found to be similar to dissimilatory Fe(III) reduction processes (Lovley, 1991; Nealson and Myers, 1992). Lovley (1993) has reviewed the dissimilatory reduction of other metals which may also be of geochemical significance including uranium, selenium, and chromium.

2.7.1 Phenols

Of the classes of compounds present in coal tar creosote, phenols have thus far been found to be the most amenable to biodegradation under anaerobic conditions. Phenol and *p*-cresol, for example, have been found to be biodegraded under nitrate-reducing, iron-reducing, sulfate-reducing, and methanogenic conditions (Londry and Fedorak, 1992). *m*-Cresol and *o*-cresol have been found to be degradable under all conditions except iron-reducing conditions. For phenol and the cresols, many metabolites produced under various anaerobic conditions have been identified and all pathways seem to involve a common benzoic acid intermediate (Londry and Fedorak, 1992). For phenol, most studies to date have shown that benzoic acid is produced as a metabolite via *para* carboxylation and subsequent dehydroxylation of phenol, although there have been a few reports of a reductive pathway in which the aromaticity of the phenolic ring is lost giving rise to cyclohexanol and further breakdown products (Londry and Fedorak, 1992). For the anaerobic degradation of cresols, substituted benzoic acids are formed either by *para*-carboxylation or methyl group oxidation. Benzoic acids are further broken down with the involvement of coenzyme A into different innocuous acids like pimelic or adipic acids, depending on the redox conditions. While much of the work on the anaerobic degradation of phenols has been studied using mixed enrichment cultures or environmental samples, there have been reports of pure organisms capable of degrading these substrates anaerobically. For example, phenol and *p*-cresol were utilized by *Geobacter metallireducens* under iron-reducing conditions (Lovley and Lonergan, 1990), phenol and *p*-cresol were degraded by a pure sulfate-reducer (Bak and Widdel, 1986b), and *o*- and *p*-cresol were metabolized by a *Paracoccus*-like strain under nitrate-reducing conditions (Rudolphi *et al.*, 1991).

A review of the literature has shown that much less is known about the anaerobic degradation of dimethylphenols. Using groundwater from a creosote-contaminated site, Flyvbjerg *et al.* (1993) found that 2,4- and 3,4-dimethylphenol were degraded under nitrate-reducing conditions, whereas 2,3-, 2,5-, 2,6-, and 3,5-dimethylphenol were resistant to biodegradation even after 12 months of incubation. Rudolphi *et al.* (1991) isolated a *Pseudomonas*-like strain which biotransformed 2,4- and 3,4-dimethylphenol to 4-hydroxy-3-methylbenzoate and 4-hydroxy-2-methylbenzoate, respectively, although these compounds were not metabolized further. In biodegradation studies conducted under methanogenic (Fedorak and Hrudey, 1984; Arvin *et al.*, 1989) and sulfate-reducing conditions (Flyvbjerg *et al.*, 1993), dimethylphenols were not degraded.

2.7.2 Polycyclic aromatic hydrocarbons

Although numerous investigations have demonstrated the anaerobic degradation of BTEX (benzene, toluene, ethylbenzenes, and *o*-, *m*-, *p*-xylene) compounds (Barker *et al.*, 1987; Grbic´-Galic´ and Vogel, 1987; Cozzarelli *et al.*, 1990; Acton and Barker, 1992; Barbaro *et al.*, 1992), few studies have examined the degradation of PAHs under anaerobic conditions, although some studies have tested for naphthalene degradation under nitrate-reducing and methanogenic conditions. In experiments conducted by Bouwer and McCarty (1983) and Kuhn *et al.* (1988), naphthalene was not degraded by denitrifying populations, whereas studies by Mihelcic and Luthy (1988) and Al-Bashir *et al.* (1990) showed that naphthalene could be degraded under nitrate-reducing conditions. The biodegradation of acenaphthene was also observed under nitrate-reducing conditions by Mihelcic and Luthy (1988). No products of degradation were reported for naphthalene or acenaphthene under these conditions. Erlich *et al.* (1982) found naphthalene to be recalcitrant in laboratory studies with creosote-contaminated aquifer material incubated under methanogenic conditions, whereas Grbic´-Galic´ (1989) detected metabolites from naphthalene degradation by methanogenic consortia initially acclimated to benzene. In this latter work, phenol was thought to be a key intermediate in naphthalene degradation, and several other aromatic and aliphatic compounds were detected in the culture fluid. Gas production was observed after 4 months of incubation, and after about 7 months, naphthalene and its transformation products were no longer detectable in the culture fluid.

2.7.3 Nitrogen-, sulfur-, and oxygen-heterocycles

Several N-heterocycles present in coal tar creosote have been shown to be biodegradable under anaerobic conditions, including indole, aniline, isoquinoline, and quinoline. The biodegradation of indole was shown to occur under methanogenic, nitrate-, and sulfate-reducing conditions. A mixed culture incubated under nitrate-reducing conditions converted indole to oxindole, isatin, isatoic acid, and anthranilic acid (Madsen and Bollag, 1989). Oxindole has also been detected as a metabolite of indole under methanogenic conditions (Berry *et al.*, 1987b; Madsen *et al.*, 1988), along with ammonium, carbon dioxide, and methane (Wang *et al.*, 1984; Bak and Widdel, 1986b). Bak and Widdel (1986a) observed the degradation of indole to ammonium and carbon dioxide under sulfate-reducing conditions by *Desulfobacterium indolicum*, a newly described species. The transformation of indole was observed under sulfate-reducing conditions by some sediment samples removed from the deep subsurface (up to 416 m below the surface) (Shanker *et al.*, 1991), and this compound was completely removed from sediment slurries under methanogenic, nitrate-, and sulfate-reducing conditions (Liu *et al.*, 1994). Schnell and Schink (1991) showed that aniline was biotransformed to 4-aminobenzoate under sulfate-reducing conditions by *Desulfobacterium anilini*.

The fate of quinoline and isoquinoline in groundwater has received considerable attention at a creosote-contaminated site near Pensacola, Florida. Groundwater samples removed from the contaminated aquifer at different distances from the source showed that quinoline and isoquinoline were present near the source, and their oxygenated analogs, 2(1H)quinolinone and 1(2H) isoquinolinone were present near the source and at the farthest sampling point away from the source (Pereira *et al.*, 1987). The authors postulated that the oxygenated analogs were anaerobic biotransformation products of quinoline and isoquinoline, and this was confirmed by laboratory cultures incubated under methanogenic conditions (Pereira *et al.*, 1987; Pereira *et al.*, 1988a). Using heavy water ($H_2{}^{18}O$), Pereira *et al.* (1988b) determined that the oxygen used for hydroxylation of quinoline came from water. 4-Methylquinoline, but not 2-methylquinoline, was also found to be biotransformed under methanogenic conditions. Using contaminated aquifer material and groundwater from the site, methane and carbon dioxide were detected in anaerobic laboratory microcosms containing quinoline, isoquinoline, and their oxygenated analogs (Godsey *et al.*, 1992). In a separate study, quinoline was completely transformed under methanogenic and sulfate-reducing conditions by sediment slurries with concommitant production of 2-hydroxyquinoline (Liu *et al.*, 1994). In the same study, less than 23% of the added quinoline was transformed under nitrate-reducing conditions. Bak and Widdel (1986b) also showed that quinoline was degraded by a mixed culture under sulfate-reducing conditions to ammonium and carbon dioxide.

Carbazole has been detected in groundwater at creosote-contaminated sites, and has also been examined for its anaerobic biodegradability. Pereira *et al.* (1987) found that carbazole was degraded under methanogenic conditions in the laboratory, although no degradation products were identified. Anaerobic studies conducted by Liu *et al.* (1994) using sediment slurries showed no evidence for the biotransformation of carbazole under denitrifying, sulfate-reducing, or methanogenic conditions.

There is very little information on the anaerobic degradation of the sulfur-heterocycles BT or DBT, although they have been reported to be biodegradable under methanogenic conditions (Arvin *et al.*, 1989; Grbic´-Galic´, 1989). Using a creosote-contaminated aquifer-derived consortium, Godsey and Grbic´-Galic´ (1989) showed that BT was susceptible to biodegradation under methanogenic conditions. Aromatic transformation products were detected in the culture fluid, including 2-hydroxythiophene, *p*-hydroxybenzenesulfonic acid, phenol, phenylacetic acid, benzoic acid, and several aliphatic compounds.

To date, there have been no studies on the anaerobic degradation of dibenzofuran, the dominant oxygen-heterocycle present in coal tar creosote. Dibenzofuran has been found to migrate through groundwater at a creosote-contaminated site (Godsey *et al.*, 1992).

2.8 Iron reduction

The coupling of organic matter oxidation with the reduction of ferric iron by bacteria has been the subject of several recent reviews (Lovley, 1987; Lovley, 1991;

Nealson and Myers, 1992; Lovley, 1993), and some points on this important process of anaerobic degradation will be highlighted here.

2.8.1 Environmental significance of microbial iron reduction

Dissimilatory ferric iron reduction has been found to greatly influence the biogeochemical cycles of carbon and other metals. The microbial reduction of ferric iron [Fe(III)] to ferrous iron [Fe(II)] has not only been studied because of its influence on iron geochemistry, but also because Fe(III) has been found to be one the most abundant terminal electron acceptors for organic matter decomposition in many aquatic sediments and subsurface environments (Lovley and Phillips, 1987). Until quite recently, even microbiologists had considered that much of the Fe(III) reduction occurring in natural environments was the result of non-enzymatic processes. This was partly due to the fact that there were no microorganisms known which could couple the complete oxidation of organic matter to the reduction of Fe(III) and obtain energy for growth. Since the early 1980s, however, microorganisms have been described which are capable of this kind of metabolism. In the iron-containing zones of aquatic sediments and aquifers, microorganisms are thought to be responsible for most of the iron reduction that occurs (Lovley *et al.*, 1991). Active iron-reducing populations have been found in deep unpolluted aquifers (Chapelle and Lovley, 1992), petroleum-contaminated shallow aquifers (Lovley *et al.*, 1994b), and shallow landfill leachate-polluted aquifers (Albrechtsen and Christensen, 1994).

Microbial Fe(III) reduction has been implicated as an important process in a number of other processes including (after Lovley, 1993):

- organic matter decomposition in a variety of freshwater, estuarine, and marine environments.
- decomposition of aromatic hydrocarbons in contaminated aquifers.

- generation of undesirably high concentrations of dissolved iron in deep pristine aquifers (greater than 0.3 mg/L) which can impair drinking water quality.
- release of toxic trace metals and phosphate.
- generation of Fe(III)-containing minerals like magnetite and Fe(II)-containing minerals like siderite and vivianite.
- corrosion of steel.

2.8.2 Effect of iron form on dissimilatory iron reduction

Dissimilatory iron reduction can be defined as the use of Fe(III) as an external electron acceptor in metabolism, which is different from assimilatory iron reduction in which Fe(III) is reduced as part of the process of iron assimilation into enzymes and cofactors. The dissimilatory reduction of iron can be simply expressed as: $Fe^{3+} + e^- \rightarrow Fe^{2+}$, but the actual reaction in soils and sediments is complex (Lovley, 1987). The Fe(III) reduced and Fe(II) produced can exist in many different soluble and insoluble forms, and it has been found that the form in which Fe(III) exists greatly influences the rate of microbial iron reduction (Lovley, 1987). For example, studies have shown that the more crystalline the Fe(III) form, the slower the rate of microbial iron reduction. Microorganisms in Fe(III)-reducing sediments were found to oxidize organic compounds using amorphous Fe(III) oxyhydroxide, but not when more crystalline forms like hematite (Fe_2O_3), akagenite (β-FeOOH), and goethite (α-FeOOH) were present (Lovley and Phillips, 1986a; Lovley and Phillips, 1986b). Poorly crystalline Fe(III) oxides were thought to be the primary sources of Fe(III) available for microbial iron reduction in sediments (Lovley and Phillips, 1987a). One study showed that much of the oxalate-extractable Fe(III) present in freshwater sediments was unavailable for microbial iron reduction (Lovley and Phillips, 1986b). The authors considered that this may be because the Fe(III) in the sediments was present as a mixed mineral with Fe(II), and thus inaccessible to microorganisms. A pure iron-reducing bacterium was found to grow faster on acetate when Fe(III) was supplied in a soluble form as Fe(III) citrate than when amorphic Fe(III) oxide was supplied (Lovley and Phillips, 1988). Magnetite (Fe_3O_4), a black, mixed ferric-ferrous mineral, accumulated in the cultures when insoluble Fe(III)oxide was provided as the Fe(III) source, whereas vivianite, a white ferrous iron mineral ($Fe_3(PO_4)\cdot 8H_20$), accumulated when the cells were incubated with soluble Fe(III) citrate. Magnetite could not be used further as an Fe(III) form by this bacterium.

2.8.3 Substrates for iron-reducers

2.8.3.1 Fermentable substrates

Microorganisms that are able to bring about the reduction of Fe(III) have been known since the late 19th century, so Fe(III) reduction as a microbial capability is not new. The first microorganisms shown to reduce Fe(III) under anaerobic conditions were those with a primarily fermentative metabolism like *Escherichia coli* and *Clostridium*, *Bacillus*, and *Lactobacillus* species (Lovley, 1987). However, none of these organisms were found to gain energy for growth from Fe(III) reduction. Fe(III) reduction is only a minor pathway for electron flow in bacteria known to reduce Fe(III) while metabolizing fermentable sugars or amino acids. It has been calculated from numerous experiments that fermentative organisms transfer less than 5% of reducing equivalents to Fe(III), therefore, Fe(III) serves as a minor electron sink for these kinds of microorganisms (Lovley, 1991). Any attempts to isolate an organism in pure culture that can obtain energy for growth by coupling the oxidation of typical fermentable substrates such as glucose to Fe(III) reduction have been unsuccessful (Lovley, 1993).

2.8.3.2 Organic acids and hydrogen

The first Fe(III)-reducing bacteria shown to gain energy for growth by coupling the oxidation of organic compounds to iron reduction were organic acid oxidizers. Using anaerobic paddy soils containing Fe(III) oxides, Kamura *et al.* (1963) showed that ^{14}C-acetate was mineralized to $^{14}CO_2$ with a concommitant accumulation of ferrous iron. Since then, mixed and pure cultures of bacteria have been shown to obtain energy for growth by coupling the oxidation of fermentation products like acetate, butyrate, propionate, ethanol, and methanol to iron reduction. For example, Lovley and Phillips (1986a) showed that these substrates were mineralized in freshwater and brackish sediment cultures under iron-reducing conditions. The first microorganism isolated in pure culture which could oxidize fermentation products under iron-reducing conditions was *Geobacter metallireducens* (Lovley *et al.*, 1987; Lovley and Phillips, 1988). This bacterium, the first member of a newly proposed genus, has been the most extensively characterized iron-reducing bacterium and much of what is known about the physiology of iron reduction is derived from studies with this bacterium (Lovley *et al.*, 1993). More characterisitics of this novel bacterium and other newly characterized species are described later. Other bacteria which have been found to couple the oxidation of fermentation products to iron reduction include *Shewanella putrefaciens* (H_2 and formate; Lovley *et al.*, 1989a), a *Pseudomonas* sp. (H_2; Balashova and Zavarsin, 1980), unidentified strain BrY (H_2; Caccavo *et al.*, 1992), *Desulfuromonas acetoxidans* (acetate, ethanol, propanol, pyruvate, and butanol; Roden and Lovley, 1993), and *Geobacter sulfurreducens* (H_2 and acetate; Caccavo *et al.*, 1994). A newly described marine bacterium, *Desulfuromonas palmifundus* (sp. nov.) has been found to obtain energy for growth by coupling iron reduction with the oxidation of acetate, lactate, succinate, fumarate, as well as with the long chain fatty acids laurate and palmitate (Coates *et al.*, 1995).

2.8.3.3. Aromatic compounds

G. metallireducens is also capable of coupling the oxidization of some aromatic compounds to iron reduction. It was first shown that this organism could grow when incubated under iron-reducing conditions with benzoate (Lovley *et al.*, 1989b). Lovley and

Lonergan (1990) further showed that toluene, phenol, and *p*-cresol were also used as sole growth substrates under iron-reducing conditions by this bacterium. *p*-Hydroxybenzoate was found to be a transient intermediate of phenol and *p*-cresol metabolism, and *G. metallireducens* was able to oxidize known catabolic intermediates of toluene (benzylalcohol and benzaldehyde) and *p*-cresol (*p*-hydroxybenzylalcohol and *p*-hydroxybenzaldehyde). Because this organism could mineralize benzoate, the authors proposed that the degradation pathways of toluene, *p*-cresol, and phenol converge at benzoate (Lovley and Lonergan, 1990), as has been suggested for the degradation of these compounds under other anaerobic conditions (Londry and Fedorak, 1992). Benzene, xylenes, ethylbenzenes, *m*-cresol, and *o*-cresol were not utilized as electron donors for this organism under iron-reducing conditions (Lovley *et al.*, 1993). To date, *G. metallireducens* is the only known iron-reducing organism available in pure culture that can oxidize and grow on aromatic compounds.

Benzene, considered to be a widespread, persistent pollutant in anaerobic environments, was found to be degraded under iron-reducing conditions in contaminated aquifer sediments when nitrilotriacetic acid (NTA) was added as an iron-chelator (Lovley *et al.*, 1994b). Under these conditions, 86% of ^{14}C-benzene added to sediments was recovered as $^{14}CO_2$. Because benzene degradation was only observed in sediments incubated with an iron chelator, it was suggested that the factor limiting the biodegradation of aromatic hydrocarbons was the poor ability of iron-reducers to access highly insoluble Fe(III) oxides in sediments. Toluene degradation rates in sediments were also enhanced when NTA was present. The rates of benzene and toluene degradation under iron-reducing conditions in the presence of NTA were comparable to those under aerobic conditions. This finding suggested that the addition of an iron chelator or chelated iron to contaminated aquifers may be a potential bioremediation strategy (Lovley *et al.*, 1994b).

2.8.4 *Geobacter* and other dissimilatory iron-reducers

A new bacterium which is capable of growth by coupling the oxidation of numerous organic acids and alcohols and monoaromatic compounds with ferric iron reduction was given the name *Geobacter metallireducens*, which means "a rod from the earth that reduces metals" (Lovley *et al.*, 1993). Using 16S rRNA sequencing, this bacterium was found to belong to the delta branch of the protobacteria, and its closest known phylogenetic relative is *Desulfuromonas acetoxidans* (Lovley *et al.*, 1993). Since this discovery, it has been found that a sulfate-reducing bacterium isolated from the marine environment can also couple the oxidation of organic compounds to iron reduction and obtain energy for growth. *D. acetoxidans*, known for its ability to reduce elemental sulfur, was also found to couple the oxidation of acetate, pyruvate, ethanol, propanol, and butanol to Fe(III) reduction (Roden and Lovley, 1993). Sulfate-reducing bacteria in salt marsh sediments were found to produce siderite (a Fe(II) mineral) via iron reduction (Coleman *et al.*, 1993). Other bacteria have recently been described which are most closely related to *G. metallireducens* including *G. sulfurreducens* (Caccavo *et al.*, 1994), *Pelobacter* species (Phillips *et al.*, 1995), and *Desulfuromonas palmifundus* (Coates *et al.*, 1995). *G. sulfurreducens* was named as such because of its ability to oxidize acetate and H_2 via Fe(III) reduction or the reduction of elemental sulfur. Thus far, this is the only known organism which can couple the oxidation of H_2 and acetate to iron reduction. *G. sulfurreducens* also uses cobalt(III), malate, and fumarate as electron acceptors during the oxidation of acetate (Caccavo *et al.*, 1994).

G. metallireducens was found to contain many of the citric acid cycle enzymes including isocitrate dehydrogenase, 2-oxoglutarate synthase, succinate dehydrogenase, fumarase, and malate dehydrogenase when acetate-grown cells were assayed for enzymes of acetate metabolism and thus it was concluded that acetate is oxidized to CO_2 via the citric acid cycle by this bacterium (Champine and Goodwin, 1991). Studies on electron transport to Fe(III) have shown that this organism contains a membrane-bound Fe(III) reductase and several soluble and membrane-bound *c*-type cyctochromes (Gorby and Lovley, 1991; Lovley *et al.*, 1993). Nitrate, Fe(III), and uranium(VI) oxidized *c*-type cytochromes in *G. metallireducens*, thus it was suggested that *c*-type cytochromes are a component of the electron transport chain(s) leading to these terminal electron acceptors in this organism. *G. sulfurreducens* also contained *c*-type cytochromes, although it was not stated whether acetate is metabolized via the citric acid cycle (Caccavo *et al.*, 1994). Recently, Caccavo *et al.* (1995) demonstrated that a single enzyme complex catalyzes the reduction of Fe(III), Co(III), and Cr(VI) in strain BrY (identified as *Shewanella alga*) and that a *c*-type cytochrome was involved in metal reduction.

2.9 Bioremediation of creosote-contaminated sites

Bioremediation can be defined as a treatment technology in which microorganisms are used to biodegrade toxic contaminant compounds into innocuous products. Although numerous chemical and physical techniques have traditionally been employed to remediate contaminated sites, bioremediation is receiving more attention as an alternative method of site remediation. As discussed above, much research in the laboratory has shown that many of the contaminant compounds in coal tar creosote are degradable by microorganisms and thus the possibility exists that these compounds will be biodegradable at contaminated sites. Although much of what is learned about aromatic compound degradation in the laboratory using ideal, controlled incubation conditions does not guarantee that the same processes will occur in a complex subsurface environment, laboratory studies can have application to field processes. For example, laboratory studies using samples from a contaminated site can determine if (1) there is a suitable microbial population present to biodegrade the contaminant, (2) the contaminants in question are biodegradable, and (3) the metabolites produced are more or less toxic than the original contaminants (the parent compounds). The following discussion outlines the rise of bioremediation as a remedial technology, the various kinds of bioremediation techniques, and case studies of successful use of bioremedation to clean-up creosote-contaminated sites.

2.9.1 The beginning of bioremediation

According to Patel *et al.* (1990) the passing of the Resource Conservation and Recovery Act (RCRA) of 1976 in the U.S. marked the beginning of the hazardous waste era. This law called upon the U.S. EPA to identify and remediate hazardous waste sites which posed a threat to human health or to the environment, which were designated "National Priorities List" (NPL) sites. The Comprehensive Emergency Response Compensation and Liability Act (CERCLA), also known as Superfund, was a law passed which governed the clean-up of old hazardous waste sites using cost-effective solutions (Patel *et al.*, 1990). For a site to be considered a Superfund site, it must be designated as an NPL site. According to Bakst (1991) there are over 1200 NPL sites in the U.S., with 30 000 more sites awaiting evaluation. Because the average cost of remediating a Superfund site using conventional methods (like incineration) has been estimated at $19 million, there has been a thrust for the development of less expensive, innovative treatment technologies (Patel *et al.*, 1990; Bakst, 1991). Bioremediation has emerged as a cost-effective treatment technology. The costs of various biological treatment processes were compared with traditional non-biological processes by Fiorenza *et al.* (1991) which exemplify the cost-effectiveness of bioremediation. Some of these estimates are listed below as cost (U.S. dollars) per cubic yard of contaminated material:

Biological	
landfarming	$\$33/\mathrm{yd}^3$
prepared bed	$\$74/\mathrm{yd}^3$
liquid-solid contactor	$\$100\text{-}150/\mathrm{yd}^3$
bioventing	$\$125\text{-}160/\mathrm{yd}^3$
in situ scheme	$\$66\text{-}123/\mathrm{yd}^3$

Non-biological	
landfill	$\$300\text{-}500/\mathrm{yd}^3$
incineration @	
1200-1800 °F	$\$200\text{-}1115/\mathrm{yd}^3$
10 000 °F	$\$1780\text{-}4460/\mathrm{yd}^3$
excavation plus above-ground incineration	$\$1000\text{-}2000/\mathrm{yd}^3$

2.9.2 Bioremediation technologies

Although bioremediation has recently entered the spotlight as an innovative remedial technology for contaminated sites, it has been used for over 30 years to remediate petroleum-contaminated soils (Ryan *et al.*, 1991). Landfarming, one of the oldest and most widely used bioremediation technologies, involves spreading out contaminated materials (like soils, sludges, or wastes) onto the land and allowing the indigenous microbial population to decompose the waste. The degradation capabilities of the microbes are generally enhanced by tilling (for increased aeration), adding nutrients (like nitrogen and phosphorus), and irrigating (to maintain moisture). This relatively simple and inexpensive technique is one of many bioremediation strategies that can be used to treat contaminated materials. This and other methods of biological treatment can generally be placed into 3 classes: solid-phase, slurry-phase, and *in situ* bioremediation (Ryan *et al.*, 1991).

2.9.2.1 Solid-phase bioremediation

Landfarming is the simplest method of solid-phase biotreatment. A more engineeered technique similar to landfarming is the use of prepared bed reactors. For this method of remediation, soil is generally excavated, and a treatment bed is prepared by fitting with a liner and irrigation, leachate collection, nutrient delivery, and possibly, inoculation systems (Ryan *et al.*, 1991; Pollard *et al.*, 1994). These treatment beds are often enclosed to control volatile emmisions. Another solid-phase method is composting, which often allows for degradation to occur at higher temperatures. In this technique, contaminated soil is mixed with a bulking agent and piled 3 to 6 feet high. Piles are aerated by turning mechanically or by forced aeration systems and moisture, pH, and nutrients are controlled (Fiorenza *et al.*, 1991; Ryan *et al.*, 1991). Composting piles can also be enclosed to control volatile emmisions. Land treatment techniques are most appropriate when contamination of DNAPLs occurs in the vadose zone (or close to the surface), when soils containing sorbed chemicals require remediation, or when DNAPLs spilled below the water table can be excavated and treated (Fiorenza *et al.*, 1991).

2.9.2.2 Slurry-phase bioremediation

This type of treatment generally involves the use of bioreactors (also known as liquid-solid contactor reactors) in which a soil slurry is treated in a controlled manner. Contaminated soil is added to an engineered, self-contained reactor which is operated in batch mode. The treatment of soil in a slurry allows for efficient mass transfer rates and maximum contact between the contaminant and microorganisms. The amount of soil that can be added to a bioreactor depends on the soil type and the amount of aeration and mixing that is possible in the system (Fiorenza *et al.*, 1991; Ryan *et al.*, 1991). Bioreactors are often seeded with organisms which are able to degrade the contaminants (Pollard *et al.*, 1994). Because slurry bioreactors are carefully controlled systems which allow for biodegradation to occur at more ideal conditions, the treatment time of waste materials is generally reduced and more degradation is often achieved compared to solid-phase and *in situ* bioremediation schemes. However, the use of bioreactors may not always be the choice remedial technology and depends on the kind of contaminant and contaminated material in question. Some disavantages of using slurry-phase bioremediation include the need to excavate and higher costs for start-up and operation (Mueller *et al.*, 1993).

2.9.2.3 *In situ* bioremediation

Bioremediation *in situ* requires the addition of nutrients and oxygen (or another terminal electron acceptor) to the subsurface to stimulate the biodegradation of contaminants by indigenous microbial populations. This method of bioremediation is usually used to treat chemicals which are dissolved in the groundwater or sorbed to subsurface sediments (Thomas and Ward, 1989). *In situ* treatment is only amenable to aquifers which have hydraulic conductivities (or permeabilities) of greater that 10^{-4} cm/sec (Thomas and Ward, 1989; Bouwer, 1992). Nutrients and oxygen are added to the subsurface via injection wells or infiltration units up-gradient of the contaminated zone, and groundwater is recovered downstream using production wells. Wells are often installed between the injection and recovery wells so that indicators of biodegradation like contaminant concentrations, cell numbers, and nutrient levels can be monitored. The groundwater can be recirculated in a closed-loop system or treated at the surface and re-injected into the subsurface (Thomas and Ward, 1989; Pollard *et al.*, 1994). Nutrients can also be injected downstream of the contaminated plume to create a zone of biological activity which will degrade contaminants as they move through this zone (Bouwer, 1992).

Oxygen is most often added as the terminal electron acceptor in *in situ* treatment because contaminants are usually most amenable to degradation under aerobic conditions. Hydrogen peroxide is frequently added because it degrades to form H_2O and O_2, providing a cheaper and more soluble source of oxygen than pure O_2 or air (Lee *et al.*, 1987). Concentrations of greater than 200 ppm H_2O_2 can be toxic to microorganisms, so caution must be used when adding it to the subsurface. Nitrate has been considered as an alternative electron acceptor to oxygen because it is more soluble and cheaper than oxygen and many compounds have been found to be degradable under nitrate-reducing conditions, although high concentrations of nitrate in groundwater can result in detrimental health effects (Thomas and Ward, 1989).

In situ biodegradation of organic compounds is difficult to prove, given the biological and non-biological processes that occur in a subsurface environment. Distinguishing between degradation of contaminants via biological means or abiotic means is a key issue for proving *in situ* biodegradation at a contaminated site (Madsen, 1991).

2.9.2.4 Choice of bioremediation strategy

The use of a particular bioremediation method at a particular site will depend on the properties of the contaminant waste, the location and extent of contamination, the properties of the site (soil and groundwater), and the goal of the remediation project (Fiorenza *et al.*, 1991). Numerous authors have stressed the need for determining the hydrogeology of a contaminated subsurface before applying any remedial method, be it chemical, physical, or biological (Lee *et al.*, 1987; Thomas and Ward, 1989; Sims *et al.*, 1990; Fiorenza *et al.*, 1991; Madsen, 1991; Bouwer, 1992). This is especially crucial if considering the application of biological remediation to a contaminated aquifer because biodegradation in the subsurface is site-specific and microsite-specific (Thomas and Ward, 1989). Thomas and Ward (1989) and Bouwer (1992) relate that the steps involved in subsurface bioremediation are (1) characterization of site hydrogeology and extent of contamination, (2) removal or free product, (3) assessing biodegradability of contaminant, (4) system design and operation, and (5) monitoring of treatment systems. The same steps were outlined by Sims *et al.* (1990) for the treatment of contaminated soils. Feasibility studies need often be conducted at the bench- or pilot-scale levels before applying a strategy to the field. Sims *et al.* (1990) and Pollard *et al.* (1994) have pointed out that the use of bioremediation strategies in conjunction with physical and/or chemical processes is likely the most effective means for attaining remediation. An example of a such a "treatment train" would be removal of free product via vacuum extraction followed by *in situ* bioremediation (Sims *et al.*, 1990).

2.9.3 Creosote-contaminated sites

Numerous bioremediation methods have been applied to treat creosote-contaminated soils and groundwaters, many of which have been successful. Ryan *et al.* (1991) list a number of Superfund sites in which creosote-contaminated materials are being bioremediated by solid-phase, slurry-phase, or *in situ* techniques.

One of the most well-characterized creosote-contaminated sites in the U.S. is the American Creosote Works Superfund site near Pensacola, Florida. Improper disposal of creosote-contaminated waste resulted in extensive contamination of surface soil and and the shallow groundwater aquifer at this site (Mueller *et al.*, 1991a). In companion papers, Mueller *et al.* (1991a and 1991b) compared the potential for using solid-phase and slurry-phase bioremediation processes at the bench-scale level. Forty-two different creosote-contaminants were monitored including phenols, PAHs, and NSO compounds. Slurry-phase bioremediation of highly contaminated sediments resulted in fast and extensive biodegradation of monitored compounds (including HMW PAHs), whereas slow losses of pollutants were observed in solid-phase experiments and more recalcitrant compounds persisted. Slurry-phase treatment of surface soils, however, resulted in slower loss of contaminants, similar to that seen with solid-phase bioremediation. On the basis of promising results with slurry-phase bioremediation at the bench-scale, research was further conducted with contaminated groundwater from the Pensacola site. Specially selected microorganisms (HMW PAH-degraders) isolated from this site were inoculated into continuous-flow bioreactors at the bench- and pilot-scale levels along with groundwater from the site (Mueller *et al.*, 1993). The results showed a decrease in concentration of creosote compounds from 1000 ppm to less than 9 ppm, and a decrease in carcinogenic PAHs from 368 ppm to 5.2 ppm. The toxicity and teratogenicity of the groundwater after treatment were reduced. Field-scale testing of this sytem showed similar results and further treatment of the bioreactor effluent using hyperfiltration reduced contamination to levels acceptable for discharge to the environment or to a sewage treatment plant (Middaugh *et al.*, 1994).

Full-scale treatment of creosote-contaminated wastes at the Southwestern Wood Preserving Superfund site in Mississippi is currently underway using 680 000-L bioreactors to treat soil slurry (Jerger *et al.*, 1991). The bioreactors are part of a treatment train which follows soil screening and soil washing processes. Using this system, indigenous microorganisms from the site biodegraded 3-ringed PAHs (99%), 4-ringed PAHs (85 to 95%), and higher-ringed PAHs (55 to 85%). Another notable slurry-phase treatment process was tested at a former wood-preserving site in Norway (Berg *et al.*, 1994). Bench- and pilot-scale studies were carried out to evaluate the treatment train processes of soil washing followed by bioslurry treatment, which showed that indigenous populations significantly degraded PAHs in the creosote-contaminated soils, but the studies were conducted using only 14 to 20% solids in the slurries. Another recent study showed that anthracene in anthracene-impregnated soil was biodegraded by an inoculated anthracene-degrading population at rates of up to 300 µg/g soil per day when high-solids content slurry (60% solids by weight) was used in rotating bioreactors (Gray *et al.*, 1994).

Feasibilty studies on the use of land treatment bioremediation have shown that solid-phase treatment can also be effective for the treatment of creosote-contaminated soils. One such study has been carried out at the J.H. Baxter International Paper Roseburg Superfund site in Weed, California, in which mostly surface soils were contaminated with creosote chemicals (McGinnis *et al.*, 1991). Using prepared treatment beds in steel boxes at bench- and pilot-scale levels, a significant portion of the PAHs present in the contaminated soil were biodegraded when nutrients were added and the soils were tilled and irrigated. Another study tested the ability of a lignin-degrading fungus, *Phanerochaete sordida*, to biodegrade PAHs in creosote-contaminated soils (Davis *et al.*, 1993).

Compared to controls, analysis of field plots inoculated with the fungus showed a large decrease in the 3- and 4-ringed PAHs, whereas the 5-ringed PAHs persisted. This was also the case with solid-phase biotreatment of creosote-contaminated soil at a site in Sweden (Ellis *et al.*, 1991). Using prepared bed reactors which were tilled, watered, and amended with nutrients, a 68% reduction of total creosote was observed after 88 days of operation, bringing the creosote concentration down from 1024 mg/kg soil to 324 mg/kg soil. Most of the compounds remaining were HMW PAHs.

Ellis *et al.* (1991) also studied the *in situ* bioremediation of creosote-contaminated soils. Following a year-long feasibility study, an *in situ* treatment system was designed and implemented to remediate soils underneath an existing structure. Groundwater was brought up to the surface, treated in bioreactors amended with PAH-degrading organisms isolated from the site, and then recirculated through the contaminated area. After 4 months of operation, 64% of the total creosote had disappeared, again, with many of the higher molecular weight compounds still present (Ellis *et al.*, 1991). The biggest limitation to existing bioremediation technologies is the persistance of HMW PAHs in both creosote-contaminated soils and groundwaters (Ellis *et al.*, 1991; Mueller *et al.*, 1993).

2.10 Research Objectives

As part of the Borden aquifer project, three different aspects of coal tar creosote biodegradation were investigated. The research objectives for this thesis were:

1. To determine the extent of aerobic biodegradation of PAHs and NSOs present in modified commercial coal tar creosote by laboratory cultures,
2. To determine whether microbial iron-reducing activity was present in Borden aquifer sediments, and

3. To study the metabolism of carbazole, a nitrogen heterocycle commonly found in coal tar creosote, by laboratory cultures which are capable of utilizing carbazole as a sole source of carbon and nitrogen, and to determine products of carbazole degradation.

The results of the investigations to address these objectives are presented in the following three chapters. As the work progressed, the most concentrated efforts were directed to the study of carbazole biodegradation and the identification of a variety of metabolites that were detected.

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3. THE AEROBIC BIODEGRADATION OF COAL TAR CREOSOTE

3.1 INTRODUCTION

Coal tar creosote has contaminated soils and groundwaters at many sites across Canada, the United States, and Europe (Mattraw and Franks, 1986; Environment Canada, 1993; Berg *et al.*, 1994). Creosote often enters these environments as a result of accidental spillage and improper handling and disposal at sites where creosote is either manufactured or used to treat wood products. Creosote, the 200-300°C distillation product of coal tar, is a diverse mixture of aromatic compounds generally consisting of about 85% PAHs, 10% phenols, and 5% NSO compounds (Mueller *et al.*, 1989). Many of the compounds present in creosote are toxic to humans and other biota and many are mutagens, teratogens, and/or carcinogens (Enzminger and Ahlert, 1987). Thus, the presence of creosote in soils and groundwater is a cause for concern especially if the contaminated groundwater is used as a drinking water source.

The fate of creosote in soil or groundwater will vary with the different properties of the compounds present in creosote, such as structure, water-solubility, reactivity, and concentration (Sims *et al.*, 1990). For example, phenols and some LMW NSO compounds are relatively water-soluble and often migrate away from the contaminant source along with the groundwater flow (Fowler *et al.*, 1994). HMW PAHs, however, are extremely water-insoluble, and will often adsorb to soil particles near the source of creosote contamination (Sims *et al.*, 1990). The fate of creosote compounds will also depend on the properties of the surface and subsurface materials that are present, including soil type and characteristics, and hydrogeological factors like porosity, permeability, and groundwater flow characteristics (Bouwer, 1992). The presence of microbial populations in soils and groundwaters with degradative capabilites as well as the presence of available terminal electron acceptors like O_2, NO_3^-, Fe(III), SO_4^{2-}, or CO_2 will also determine the fate of creosote compounds.

Microorganisms are capable of degrading phenols, PAHs, and NSO compounds under aerobic and anaerobic conditions (Berry *et al.*, 1987; Arvin *et al.*, 1989; Cerniglia, 1992). Although an increasing number of reports is emerging which describe the degradation of aromatic compounds under various anaerobic conditions, much more is known about the aerobic degradation of many of these compounds which has been the subject of several reviews (Cerniglia, 1984; Gibson and Subramanian, 1984; Cerniglia, 1992). Mixed or pure cultures of bacteria and fungi have been shown to biodegrade phenols, PAHs composed of 2 to 4 aromatic rings, and several NSO compounds prevalent in creosote incuding quinoline, carbazole, dibenzothiophene, and dibenzofuran. It has generally been found that lower molecular weight PAHs are more readily biodegradable than higher molecular weight PAHs. For example, metabolic pathways have been elucidated for several 2- and 3-ringed PAHs, whereas few metabolites have been identified for compounds containing 4 or more aromatic rings (Cerniglia, 1992). PAHs are generally biodegraded via initial attack of a dixoygenase enzyme in which molecular oxygen is stereoselectively added to adjacent carbon atoms in an aromatic ring, giving rise to a *cis*-dihydrodiol, usually in the 1 and 2 positions. A dehydrogenase then transfers reducing equivalents to NAD(P) with subsequent restoration of aromaticity. The 1,2-dihydroxy metabolite which results may then be cleaved in an *ortho* or *meta* fashion depending on the bacterium (Gibson, 1993). In many cases, these dioxygenases are not substrate-specific, and one bacterium is often capable of oxidizing a wide-array of aromatic substrates (Strubel *et al.*, 1989; Heitkamp and Cerniglia, 1989; Walter *et al.*, 1991; Monna *et al.*, 1993; Boldrin *et al.*, 1993). It has recently been shown that fluorene and dibenzofuran are oxidized via dioxygenase attack in the unusual angular position, which deviates from the traditional 1,2-mode of oxidation (Engesser *et al.*, 1989). Carbazole degradation is proposed to proceed via angular dioxygenation as well (Ouchiyama *et al.*, 1993).

Because microorganisms are capable of biodegrading numerous creosote compounds, the exploitation of microbial populations to remediate creosote-contaminated sites has received increased attention (Ryan *et al.*, 1991). Bioremediation, the process by which toxic compounds are degraded by microorganisms to innocuous products, is advantageous over traditional physical and chemical remediation because it is a more cost-effective method of site clean-up (Fiorenza *et al.*, 1991). There are numerous different bioremediation technologies which have been developed ranging from simple landfarming to the use of sophisticated, engineered *in situ* treatment systems and above-ground bioreactors. For most biological remediation technologies, microbial activity is enhanced by aeration and the addition of nutrients (Thomas and Ward, 1989). Some bioremediation schemes exploit microbial populations indigenous to a contaminated site, whereas others use specific microorganisms known to have the enzymatic capabilities to biodegrade the contaminants in question.

Before a bioremediation scheme can be implemented, numerous factors must be considered to determine its feasibility at a contaminated site. These include determining the hydrogeology of the site, the properties of the contaminant, the location and extent of contamination, as well as assessing the biodegradability of the contaminant (Bouwer, 1992). Bioremediation has been deemed feasible at several creosote-contaminated sites involving land treatment, *in situ* schemes, and above-ground bioreactors (Ryan *et al.*, 1991).

In order to study the fate of creosote compounds in subsurface environments, a 5-year field experiment was set up in 1991 at a well-characterized shallow, sandy aquifer located at CFB Borden, Ontario. A known amount of commercial coal tar creosote was emplaced below the water table of this aquifer, and the removal of groundwater and core samples near the source and at selected points within the contaminated plume is ongoing in order to evaluate the persistence and migration of creosote compounds over time. Several laboratory and field experiments are underway to determine various aspects of creosote compound migration and attenuation, toxicity, biodegradation, and chemical and biological remediation. One of the objectives of these studies was to examine the aerobic biodegradation of the compounds present in this commercial coal tar creosote. This chapter presents results on the biodegradation of this commercial coal tar creosote under aerobic conditions by mixed microbial populations, some of which were enriched from creosote-contaminated soil.

3.2 MATERIALS AND METHODS

See Appendix A for media compositions and sources of all chemicals used in these experiments.

3.2.1 Characterization of creosotes used

Laboratory cultures were initially set up on coal tar from Sidney, Nova Scotia. Compounds in Sidney coal tar were identified based on gas chromatography-mass spectrometry (GC-MS) analysis by geochemists at the Institute for Sedimentary and Petroleum Geology (ISPG), Geological Survey of Canada, Calgary, Alberta. The coal tar creosote used for the majority of biodegradation studies was commercial coal tar creosote from Ontario (denoted OCT). The compounds present in OCT were also identified by GC-MS analysis at ISPG. Fractionation of OCT was performed using open column chromatography (Fowler *et al.*, 1991) to obtain saturate, aromatic, and resin fractions. Aromatic hydrocarbons (82.5%) predominated in OCT, with lower amounts of resins (15.2%) and negligible amounts (0.8%) of saturates present (Fowler *et al.*, 1994).

In order to confirm the chemical composition of OCT, a different fractionation scheme on an activated silica gel column to obtain saturate, aromatic, and polar fractions was carried out in our laboratory. To prepare the column, 8 g of activated silica gel was added as a slurry in dichloromethane (CH_2Cl_2) to a 1.1 cm ID X 30 cm long column plugged with a small amount of glass wool. Silica gel was allowed to settle, and 0.5 mL sea sand was added to the top. CH_2Cl_2 was drained to the top of the sand, and *n*-pentane was added to displace the CH_2Cl_2 in the column, which was then ready for chromatography. To prepare the sample to be analyzed, 50 µL OCT in CH_2Cl_2 was mixed with unactivated silica gel to dry off the solvent. The sample, having the texture of sand, was then added to the column, and about 1 cm of sand was added on top of this. The column was then developed sequentially with 5 mL *n*-pentane, 20 mL 20% CH_2Cl_2 in *n*-pentane, 25 mL 50% CH_2Cl_2 in *n*-pentane, and 30 mL 50% benzene in methanol. The first 12 mL collected were discarded, the second 12-14 mL contained the saturates, the third 20-30 mL contained the aromatics, and the fourth contained the polar compounds. Samples were concentrated by rotary evaporation before GC analysis.

Differential extractions with CH_2Cl_2 were also performed to separate OCT into phenolic, PAH and S- and O-heterocyclic, and N-heterocyclic fractions using a scheme outlined in Mueller *et al.* (1991a). In addition, simple acidic and basic extractions with CH_2Cl_2 of whole OCT were done, and a comparison of the GC peaks obtained using each separation scheme was made.

3.2.2 General culturing methods

3.2.2.1 Initial enrichments on Sidney coal tar

Long-maintained laboratory oil-degrading cultures were initially set up (January, 1991) to test for the biodegradability of Sidney coal tar. These included SL, which was enriched from aviation fuel-contaminated beach material from Shell Lake, Northwest Territories, and ESSO AG and ERN BIO which were enriched from activated sludge samples from wastewater treatment systems at two petroleum refineries in Germany (Fedorak and Peakman, 1992). SL had been maintained by monthly tranfers to fresh medium containing Prudhoe Bay (PB) crude oil as the sole carbon source since 1983, and ERN BIO and ESSO AG were likewise maintained since 1986. All cultures were set up in

500 mL Erlenmeyer flasks containing 200 mL of mineral medium, denoted B+N (see Appendix A). Because Sidney coal tar was very thick and difficult to pipet, it was added to sterile, empty flasks in a solution of CH_2Cl_2 such that 1.0 mL contained 0.2 g of coal tar. The CH_2Cl_2 was allowed to evaporate before 200 mL sterile B+NP medium was added. The flasks were then inoculated with 20 mL of SL, ESSO AG, or ERN BIO grown on PB oil. Soil (10 g) collected from an oil spill near Rocky Mountain House, Alberta in August, 1990 (denoted RMH Soil) was also set up as a source of aromatic-degraders for Sidney coal tar. Cultures were incubated in the dark at 28°C on a rotary shaker at 200 rpm and were transferred every 3 to 4 weeks. The four cultures were maintained on Sidney coal tar for 5 transfers (5 months) before being transferred into fresh medium containing OCT as the sole carbon source.

3.2.2.2 Enrichments on Ontario coal tar creosote

Because OCT was easy to pipet, 0.05 mL was added directly to 200 mL sterile B+NP in 500-mL Erlenmeyer flasks using a sterile 1.0 mL pipet. SL, ESSO AG, ERN BIO, and RMH Soil previously incubated with Sidney coal tar were transferred, in duplicate, to flasks containing OCT (20 mL transferred) and incubated as above. Later, 15 mg carbazole and 30 mg phenol were added to one of each duplicate culture in addition to OCT and these duplicates were then permanently maintained with these additional substrates.

In addition to the cultures originally set up on Sidney coal tar, three soil samples were obtained from a creosote-contaminated site in Edmonton, Alberta to set up on OCT. These soil samples were of varying degrees of contamination: "lightly"-, "medium"-, and "heavily"- contaminated. Soxhlet extractions (with CH_2Cl_2) and plate counts (using the spread plate method on plate count agar [PCA]) were performed for each of the soil samples to examine the degrees of contamination and presence of microorganisms, respectively. The "lightly"-contaminated soil had low numbers of bacteria and GC analysis of its Soxhlet extraction did not show many contaminants, so this soil was not used for creosote-degradation studies. Soxhlet-extracted "medium"- and "heavily"-contaminated soils showed many contaminants by GC as well as high numbers of bacteria, so these soils were used for creosote-degradation studies and were given the names DT Medium and DT Heavy. As well, a mixture of these two soils was used to set up a creosote-enrichment culture referred to as DT Mixed. For each of DT Medium and DT Heavy, 7 g of soil was added to 200 mL B+NP medium containing 0.05 mL OCT. For the DT Mixed culture, 5 g of each soil was added. The cultures were routinely incubated in the dark at 28°C with shaking unless otherwise noted and were maintained by transferring 20 mL every 3 to 4 weeks into fresh medium and substrate.

3.2.3 Extractions of cultures

Cultures incubated with Sidney coal tar or OCT were extracted under acidic and basic conditions with CH_2Cl_2 to look for loss of creosote components. Cultures originally containing soil as inoculum were tranferred twice before being extracted. After each transfer, the spent culture medium was acidified to $pH \leq 2$ with 2 mL 4N H_2SO_4, and extracted in a separatory funnel with four 20-mL volumes of CH_2Cl_2. Then, the aqueous layer was made basic to $pH \geq 10$ with 4 mL 4N NaOH and extracted with three 20-mL volumes of CH_2Cl_2. The organic layers were dried over Na_2SO_4 and concentrated to small volumes by rotary evaporation. The samples were quantitatively transferred to 1 dram vials and concentrated under a gentle stream of nitrogen to an appropriate volume for GC analysis. All samples in an experiment were concentrated to the same volume for a more accurate comparison of peak sizes in GC chromatograms. A reference compound, *n*-hexadecane, was added prior to extraction under acidic conditions. For experiments to determine the extent of removal of compounds in OCT, the ratio of the peak area of a given compound to the peak area of *n*-hexadecane was calculated and compared to that in the sterile control, and from this, percent removal was calculated for each compound monitored.

3.2.4 Experiments with radiolabelled substrates

An experiment examining the mineralization over time of various compounds in coal tar creosote was performed in 500-mL Erlenmeyer flasks equipped with side arms for sampling $^{14}CO_2$ (Fedorak *et al.*, 1982). DT Mixed (20 mL) was inoculated into flasks containing 200 mL B+NP, 0.05 mL OCT, and one of the following radiolabelled compounds in OCT: [U-^{14}C]phenol, [1-^{14}C]naphthalene, [U-^{14}C]biphenyl, [9-^{14}C]phenanthrene, [9-^{14}C]anthracene, and [U-^{14}C]carbazole. ^{14}C-Substrates in OCT were added such that approximately 10 000 dpm was present in 5 mL of culture solution. Cultures were incubated and sampled at various times up to 25 days to analyze for the presence of $^{14}CO_2$. Liquid (5 mL) and headspace air (10 mL) were sampled from the side arms using sterile syringes and added to previously evacuated 60-mL serum bottles. The contents of the serum bottles were acidifed and flushed into scintillation vials containing 10 mL ACS fluor and 1 mL Carbo-Sorb II to trap $^{14}CO_2$ using the apparatus described by Fedorak *et al.* (1982). The amount of $^{14}CO_2$ was then determined by liquid scintillation counting. The percentage of label recovered as $^{14}CO_2$ was calculated based on the original amount of label present in 5 mL of liquid culture.

Another mineralization experiment designed to test the ability of OCT-degrading cultures to degrade the saturated and aromatic compounds in PB crude oil used 158 mL serum bottles capped with rubber stoppers. DT Mixed, DT Medium, and SL cultures that had been maintained with OCT as a sole carbon source for 4 years, as well as SL maintained on PB oil (since 1983), were inoculated (1 mL) into 10 mL B+NP. PB oil (10 µL) containing either ^{14}C-*n*-hexadecane or ^{14}C-phenanthrene was added (such that 10 µL contained about 10 000 dpm of the appropriate substrate) and the cultures were incubated for 7, 14, or 28 days with shaking at 28°C. At each of these times, a serum bottle for each culture and substrate was acidified, flushed, and counted to determine the percentage of label recovered as $^{14}CO_2$. As well, shake flask cultures were incubated simultaneously with 0.2 mL PB oil and extracted with CH_2Cl_2 under acidic conditions after 7, 14, and 28 days. The organic extracts were fractionated into saturate and aromatic fractions to determine the loss of compounds in these fractions by the various cultures. Sterile controls were also established.

3.2.5 Plating of cultures

Colonial morphologies from the creosote-degrading cultures were observed on Plate count agar (PCA), and growth of the creosote-degrading cultures was determined using the plate count method. To detect the presence of specific hydrocarbon-degraders, 10-fold dilutions of some of the creosote-degrading cultures were plated on minimal medium plates, then sprayed with solutions of phenanthrene, anthracene, or pyrene (in ether or CH_2Cl_2) (Foght and Westlake, 1988). Naphthalene was added as crystals to the lids. The plates were incubated at 28°C for up to 2 months, and were periodically examined for growth and clearing of substrate.

Another method used to test for hydrocarbon-degraders involved adding the appropriate substrate in dimethyl sulfoxide to hot minimal medium before the plates were poured in an attempt to get an even distribution of substrate crystals throughout the plates. Serial 10-fold dilutions of creosote-degrading cultures were then plated, incubated, and examined for growth in addition to that seen on background plates.

3.2.6 Anaerobic experiments with OCT

Water-soluble OCT compounds were extracted from whole OCT by shaking 10 mL OCT in 100 mL H_2O in a flask for 24 h. The water layer was then used as a source of water-soluble OCT substrates. The acidified extract of the water-soluble fraction contained phenols, acenaphthene, fluorene, and phenanthrene and the basic extract contained quinoline, isoquinoline, and indole (compounds identified by M.G. Fowler at ISPG). Anaerobic sewage sludge from the Gold Bar Waste Water Treatment plant in Edmonton, Alberta was used as inoculum to see if the water-soluble OCT compounds were degradable under methanogenic conditions. Using strict anaerobic technique, a mineral salts medium was prepared (WR86 medium, Appendix A) and 1 mL was dispensed into 60 mL serum bottles, which were then capped, sealed, and autoclaved. After sterilization, Na_2S was added as a reducing agent. The bottles were inoculated with 4 mL of sewage sludge and various amounts of water-soluble OCT substrates were added ranging from 0 to 50% substrate by volume. O_2-free H_2O was added to controls and to make the volumes in the bottles equal. Triplicate bottles were set up for each substrate concentration. The cultures were incubated at 35°C and monitored for methane production by GC. Another experiment was set up in the same way except that varying amounts of whole OCT was added as substrate, ranging from 0 to 8.3% substrate by volume. Methane production was also monitored for these cultures.

3.2.7 Analytical methods

Acidic and basic extracts from creosote-degrading cultures were analyzed by GC using a Hewlett-Packard (HP) model 5390A gas chromatograph equipped with a flame ionization detector (FID). Injector and detector temperatures were set at 250°C. The temperature program routinely used for a DB-5 capillary column (30 m in length, 0.25 mm internal diameter, 0.25 μm film thickness) was 90°C held for 2 min, increasing at 4°C per min to 250°C, then held for 4 or 16 min. Methane analyses were performed on a HP model 5700 GC equipped with a FID at 200°C. A 6-foot glass column was used, packed with Chromosorb 104 (80/100) (Supelco). The injection port and oven temperatures were at 25°C. Standards containing a known percentage of methane were prepared and run before sample analyses on a given day in order to calculate the percent CH_4 produced in the cultures. HP model 3390A integrators were used to obtain peak areas of various compounds.

A Beckman Model LS 3801 liquid scintillation counter was used to determine the amount of $^{14}CO_2$ recovered in mineralization experiments.

3.3 RESULTS

3.3.1 Sidney coal tar

SL, ERN BIO, ESSO AG, and RMH Soil cultures were tested for their abilities to biodegrade compounds present in Sidney coal tar which included phenols, LMW and HMW PAHs, and NSO compounds. Three of these cultures, SL, ERN BIO, and ESSO AG, had been maintained on PB crude oil which is composed predominantly of saturated hydrocarbons, but contains some PAHs. Exposing these cultures and an oily soil to Sidney coal tar was an attempt to acclimate these populations to a mixture which contained mostly aromatic compounds. During incubation, these cultures became golden in color, compared to an uninoculated sterile control which was clear. An examination of the cultures on PCA showed numerous different colonial morphologies for each culture. Typically, after 1 month of incubation, the numbers of colony forming units ranged from 8 X 10^6 to 2 X10^7 per mL of culture supernatant. Using a 10% inoculum, the numbers in the cultures increased approximately 10-fold during a month of incubation with Sidney coal tar.

After each monthly incubation, the four culture supernatants and a sterile control were extracted with CH_2Cl_2 under acidic and basic conditions and the organic extracts were analyzed by GC to observe the qualitative loss of coal tar compounds. After the first two incubation periods, only the loss of phenols and methylphenols was observed in the acidic extract of each culture, as well as a decrease in the peak sizes of compounds present in the basic extracts. In subsequent 1-month incubation periods, naphthalene,

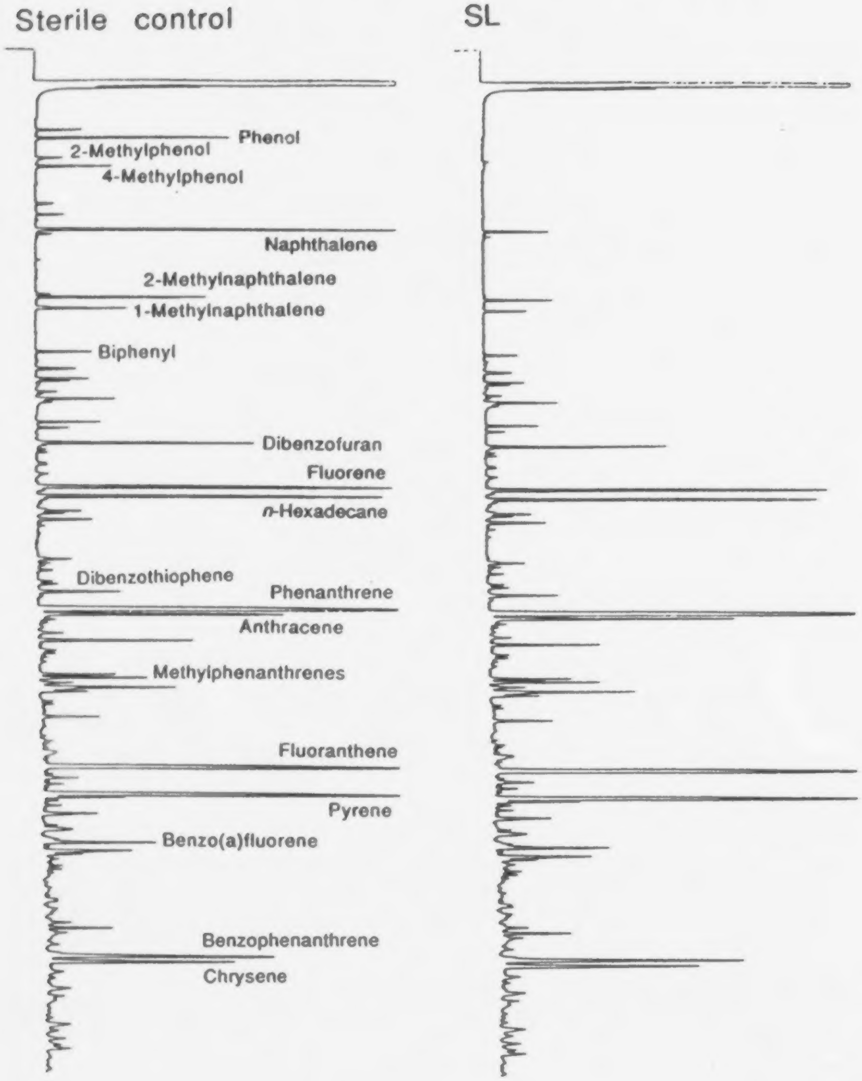


Figure 3.1 GC chromatograms of acidified supernatant extracts of a sterile control and the SL culture incubated with Sidney coal tar for 1 month.

methylnaphthalenes, biphenyl, and dibenzofuran peaks decreased in size as well. Figure 3.1 shows chromatograms of residual Sidney coal tar extracted from a sterile control and from an active SL culture after incubation for 1 month. While the peak sizes of some 1-, 2- and 3-ringed compounds decreased in the SL chromatogram compared to the sterile control, no decreases were seen for phenanthrene, anthracene, or compounds with 4 or more aromatic rings. In contrast, the basic extracts, containing predominantly quinoline, isoquinoline, and indole, showed a loss of all compounds in the cultures compared to sterile controls (data not shown).

For these initial experiments, 0.2 g of Sidney coal tar was added to each culture. Because very little removal was observed, it was thought that this concentration of coal tar might be toxic to the populations. Therefore, subsequent cultures were incubated in the presence of 0.1 g and 0.05 g of Sidney coal tar, and loss of compounds was examined by GC. Incubation of the 4 cultures with lower concentrations of Sidney coal tar did not result in increased loss of peaks. Thus, SL, ERN BIO, ESSO AG, and RMH Soil were only able to biodegrade or biotransform some low-molecular-weight compounds present in Sidney coal tar. After 5 monthly incubations with Sidney coal tar, these four cultures were tested for their abilities to remove the compounds present in Ontario coal tar creosote.

3.3.2 Ontario coal tar creosote (OCT)

3.3.2.1 Chemical analysis of OCT

The fractionation scheme carried out in our laboratory resulted in a GC chromatogram for the aromatic fraction which was similar to that obtained using a different fractionation protocol by geochemists at ISPG, although no attempt was made in our laboratory to quantitate the amounts of compounds in OCT. Virtually no peaks were seen in the saturate or polar fractions that were collected in our laboratory. The differential extraction of OCT resulted in a PAH/O-, S-heterocycle chromatogram which was similar to the chromatogram obtained by a simple acid extraction, while the N-heterocyclic chromatogram closely matched that obtained by a basic extraction of OCT. The phenolic fraction obtained by differential extraction contained phenol, methylphenols, dimethylphenols, and other unidentified peaks. Phenols such as these could also be extracted under acidic conditions when added to whole OCT. Because many aromatic compounds present in OCT could be detected by GC after extraction with CH_2Cl_2 under acidified or alkaline conditions, which was much simpler and less time consuming for routine analysis than fractionation or differential extraction, this was the procedure used to determine the degradation of creosote compounds by cultures and controls. A chromatogram of an acidified extract of a sterile control incubated with OCT is shown in Fig. 3.2. The compounds labelled on this chromatogram (identified by GC-MS by Fowler *et al.*, 1994) were monitored in the creosote biodegradation studies presented here.

3.3.2.2 Qualitative OCT analysis

Seven laboratory mixed cultures were monitored over time for their abilties to biodegrade compounds present in OCT. For qualitative studies, cultures and a sterile control were incubated for 2 to 4 weeks, then extracted under acidified and alkaline conditions and analyzed by GC. Compounds present in the basic extracts of the sterile controls were no longer detected by GC analysis of the extracted culture supernatants after 2 weeks of incubation, however, the loss of compounds present in the acidified extracts varied with each culture. Representative acidified extract chromatograms from a sterile control and each of the seven cultures after 3 weeks of incubation are shown in Fig. 3.3 (DT cultures) and Fig. 3.4 (SL, ERN BIO, ESSO AG, RMH Soil). In all cultures, the methylnaphthalenes, biphenyl, and the dimethylnaphthalenes had essentially disappeared. Acenaphthene, dibenzofuran, and fluorene were removed to some extent by all of the cultures, although acenaphthene was not removed in the RMH Soil or DT Heavy cultures, and fluorene was not lost in the latter culture, either. Loss of the methyldibenzofurans and

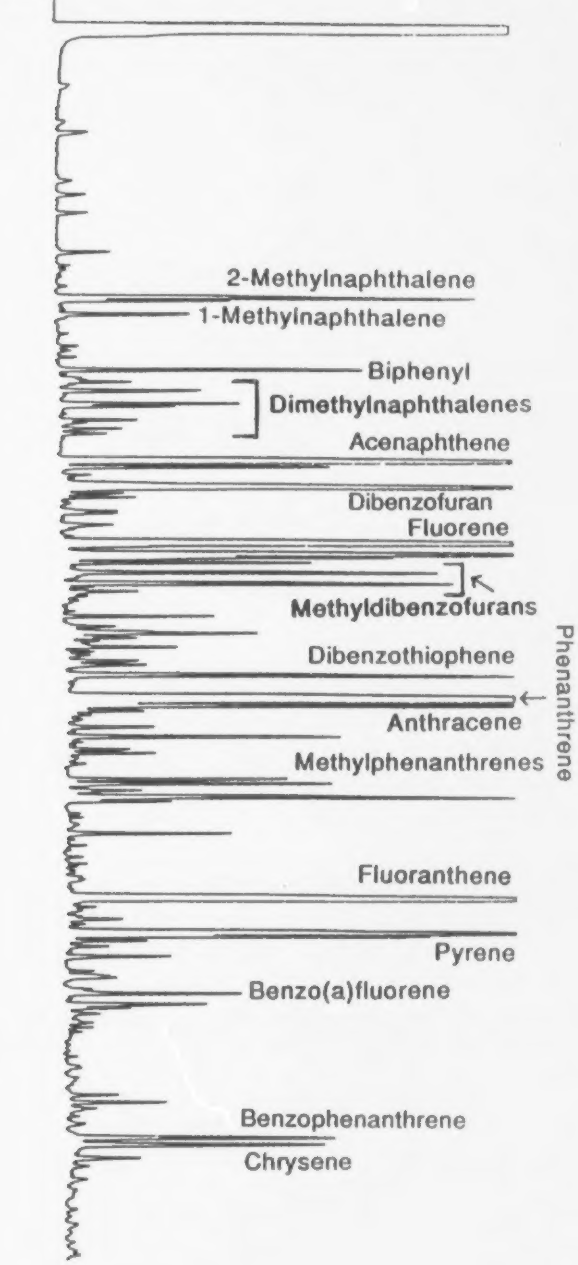


Figure 3.2 Compounds identified by GC-MS in the acidified extract of sterile B+N medium containing OCT.

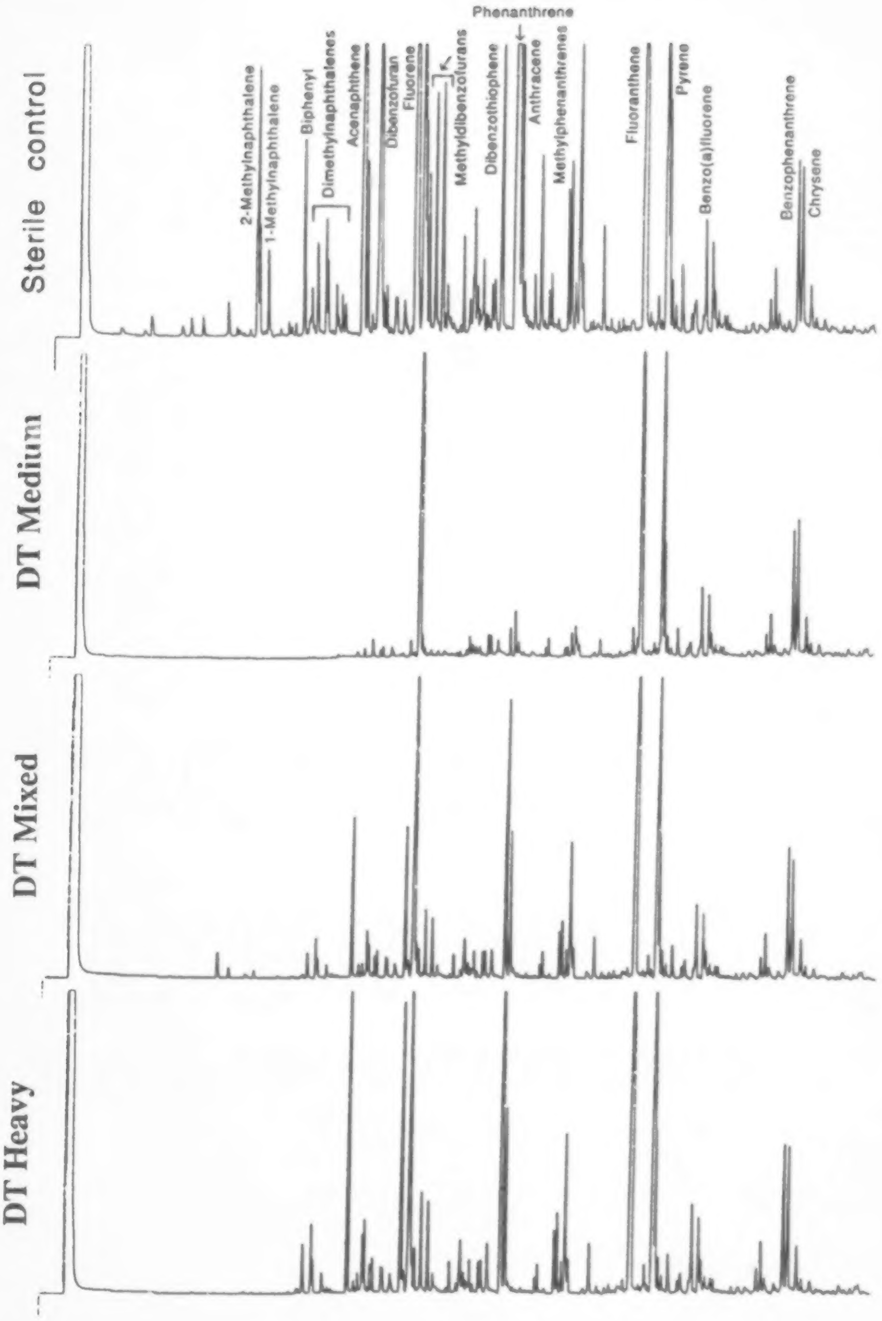


Figure 3.3 A comparison of the chromatograms of the acidified supernatant extracts of a sterile control and the three DT cultures incubated with OCT for 3 weeks.

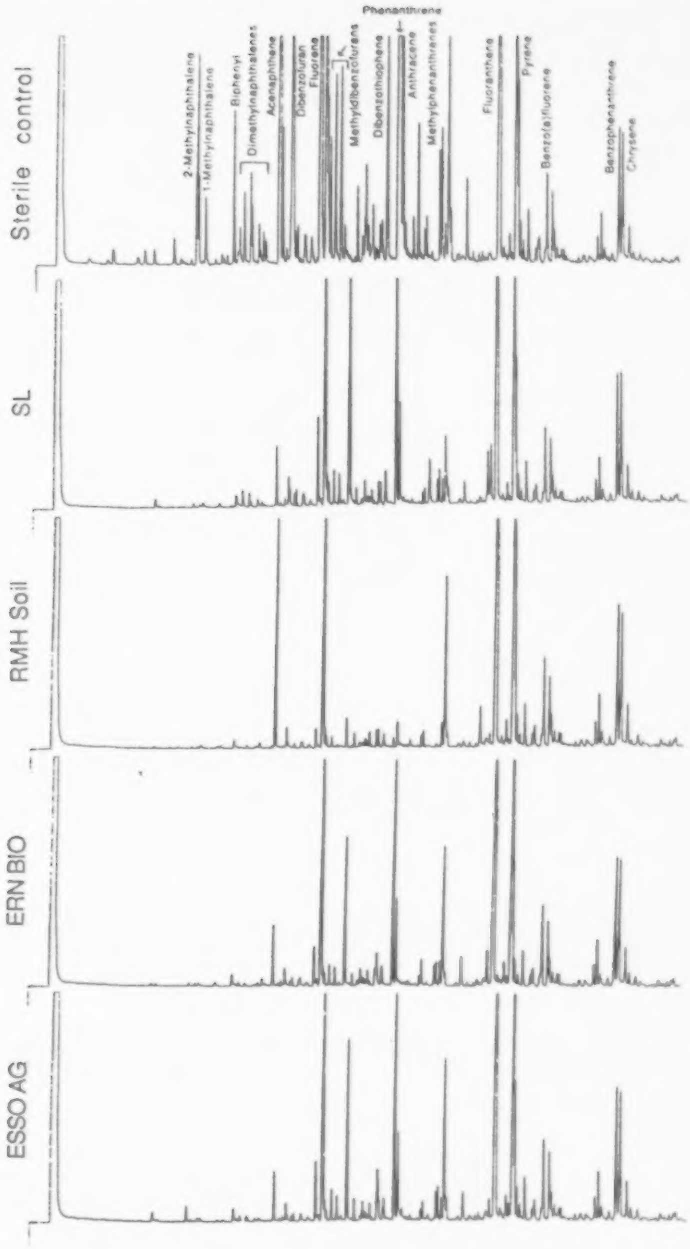


Figure 3.4 A comparison of the chromatograms of the acidified supernatant extracts of a sterile control and the SL, ERN BIO, ESSO AG, and RMH Soil cultures incubated with OCT for 3 weeks.

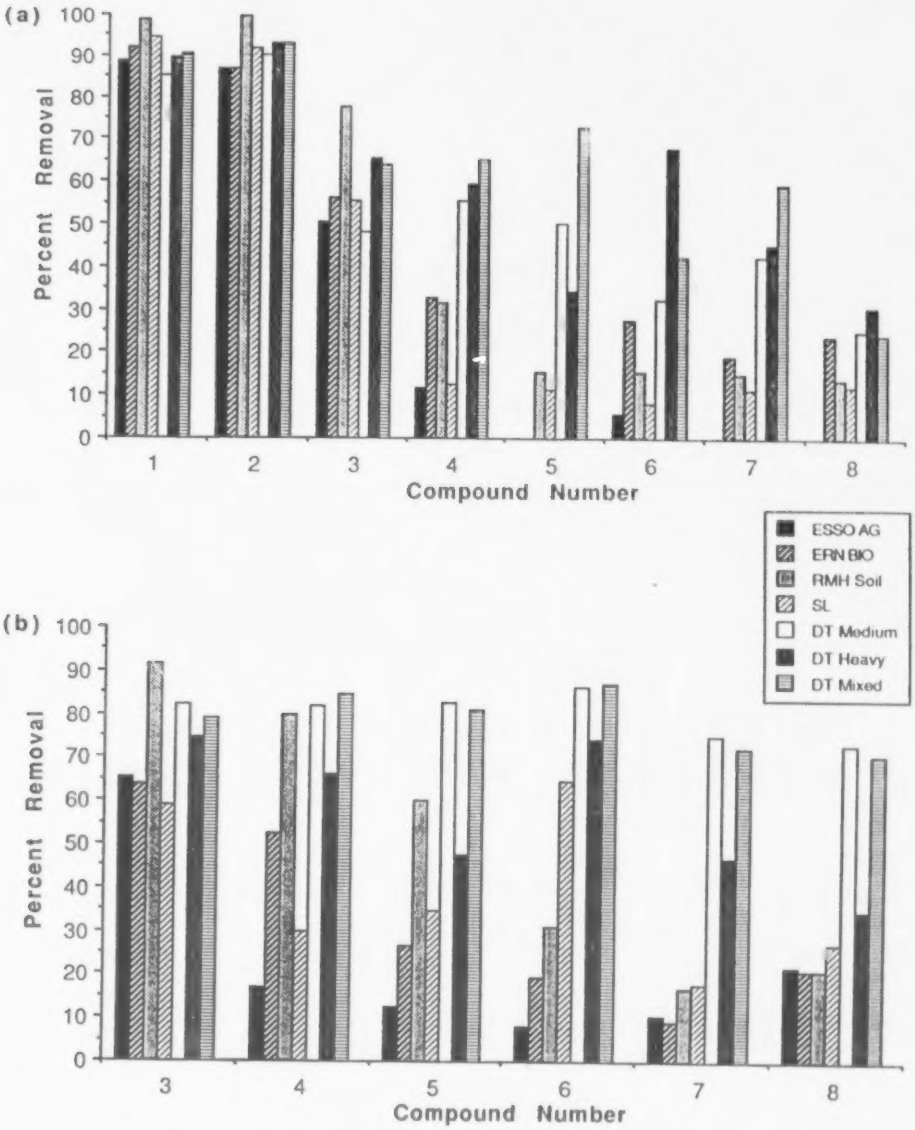
dibenzothiophene was evident in all of the cultures and decreases in the sizes of the phenanthrene and anthracene peaks were seen as well. In the RMH Soil culture, phenanthrene had almost completely disappeared, and complete loss of both phenanthrene and anthracene was observed in the DT Medium culture. The methylphenanthrene peaks decreased in size for all of the cultures, but the fluoranthene, pyrene, benzo(a)fluorene, benzophenanthrene, and chrysene peaks did not compared to the sterile control.

3.3.2.3 Extent of removal of compounds in OCT

The extent of removal of several compounds in OCT by all seven cultures was monitored periodically for over 3 years, from February, 1992 until July, 1995. The losses of carbazole and phenol were also monitored in the SL, ERN BIO, ESSO AG, and RMH Soil cultures when OCT was amended with these compounds. Cultures were usually transferred every 4 weeks and extracted under acidic conditions after selected transfers. At times, the cultures were incubated for 8 weeks before being transferred to compare the losses of compounds after 8 weeks with those after 4 weeks. The removal of the aromatic compounds labelled in Figure 3.2 was regularly monitored. Percent removal of each compound was calculated with respect to sterile controls, based on the compound to *n*-hexadecane peak area ratios from GC chromatograms.

3.3.2.3.1 OCT as the sole carbon source

Figure 3.5 shows the percent removal of many of the compounds monitored in all seven cultures incubated with OCT for 4 and 8 weeks. These results were obtained by calculating the average percent removal of each compound from 4 week or 8 week incubation experiments conducted over time (4 experiments for 4 week analyses, 2 experiments for 8 week analyses). With few exceptions, the percent removal of a particular compound in a particular culture was consistent over time. For example, the percent removal of fluoranthene by the ERN BIO culture after 4 weeks of incubation in 1993



1. Fluorene
2. Phenanthrene
3. Anthracene
4. Fluoranthene
5. Pyrene
6. Benzo(a)fluorene
7. Benzophenanthrene
8. Chrysene

Figure 3.5 The average percent removal of 3- and 4-ringed PAHs in creosote by seven mixed creosote-degrading populations incubated with OCT for (a) 4 weeks or (b) 8 weeks.

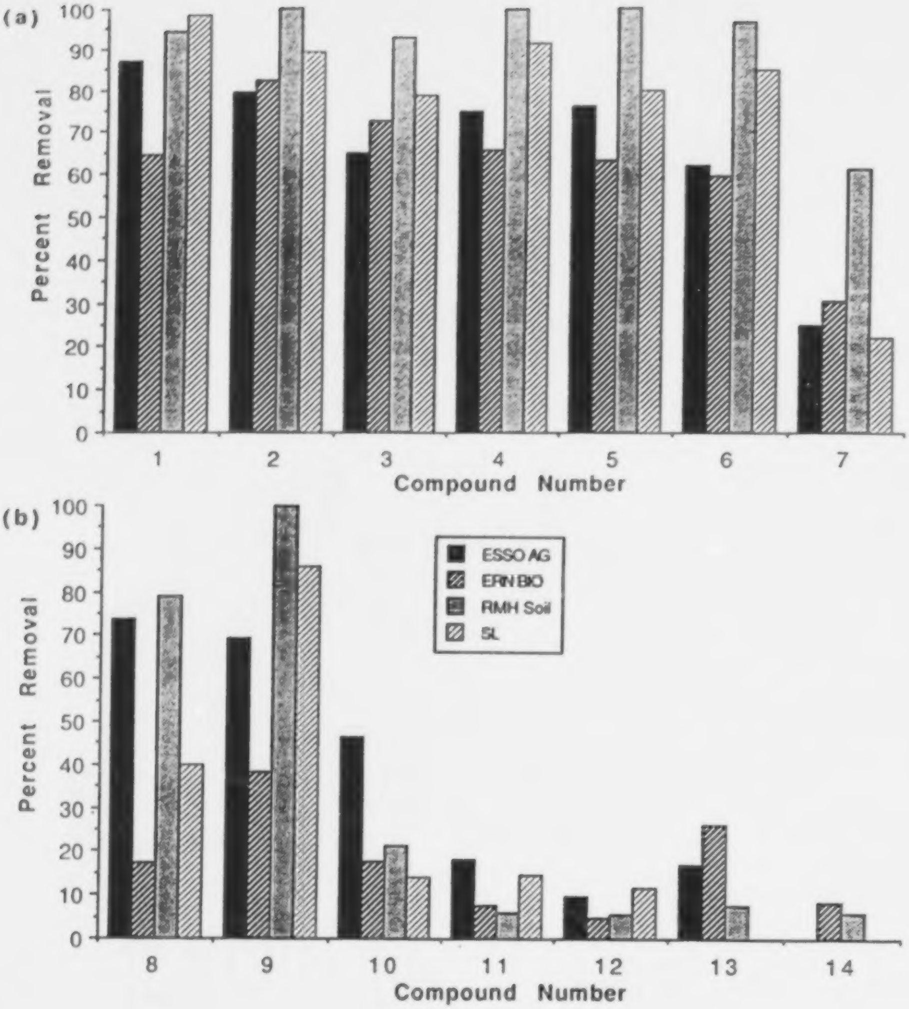
(53%) was similar to that after 8 weeks of incubation in 1995 (49%). Therefore, because the data were consistent, it was possible to calculate the average percentage removal of each compound with validity.

Only those compounds which did not show 100% removal are shown in the bar graphs in Fig. 3.5. Using the methods described above, complete removal of the methylnaphthalenes, acenaphthene, dibenzofuran, the methyldibenzofurans, dibenzothiophene, and the methylphenanthrenes was observed in all of the cultures after 4 weeks of incubation. Greater than 85% removal of fluorene and phenanthrene was evident in all cultures after 4 weeks, while anthracene loss ranged from 45-65% in all cultures except for RMH Soil in which 77% of the anthracene present was lost (Fig. 3.5a). Much less removal of fluoranthene, pyrene, and other 4-ringed compounds was evident, although there was more loss of these compounds in the DT soil enrichment cultures than in the others. For example, 55 to 65% fluoranthene was removed in the DT cultures but only 11 to 32% was lost in the other four cultures after 4 weeks.

After 8 weeks of incubation, the complete removal of fluorene and phenanthrene was observed in all of the cultures (thus not shown in Fig. 3.5b). The RMH Soil culture removed 91% of the anthracene present after 8 weeks, and anthracene was further removed in the other cultures with prolonged incubation as well. Fluoranthene and pyrene were also removed to greater extents, and this was most evident in the DT Mixed and DT Medium cultures in which greater than 80% of these compounds were lost after 8 weeks of incubation. Overall, the 4-ringed compounds were removed to the greatest extent by the DT soil enrichment cultures. The RMH Soil culture contained organisms most able to biodegrade compounds in OCT of all the cultures originally exposed to Sidney coal tar.

3.3.2.3.2 OCT, carbazole, and phenol as carbon sources

Figure 3.6 shows the percent removal of carbazole and selected OCT compounds by the SL, ERN BIO, ESSO AG, and RMH Soil cultures when incubated with OCT that

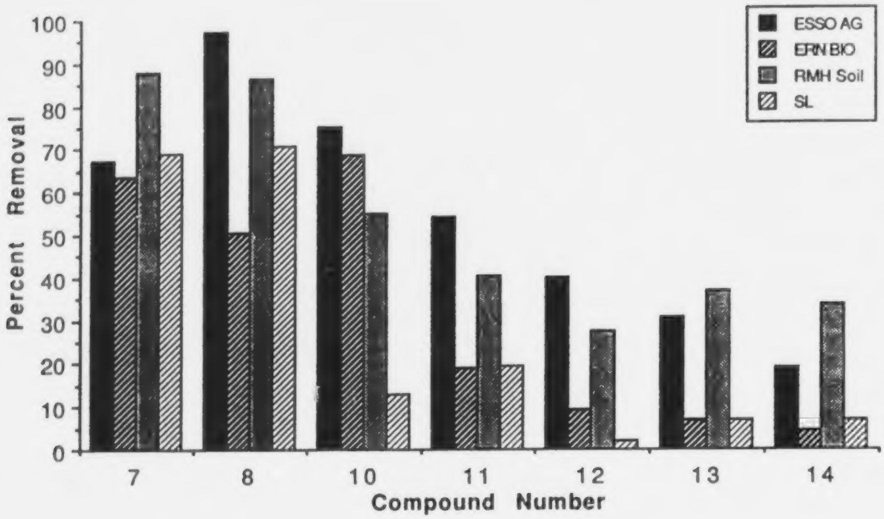


1. Acenaphthene
2. Dibenzofuran
3. Fluorene
4. Methyldibenzofurans
5. Dibenzothiophene
6. Phenanthrene
7. Anthracene
8. Carbazole
9. Methylphenanthrenes
10. Fluoranthene
11. Pyrene
12. Benzo(a)fluorene
13. Benzophenanthrene
14. Chrysene

Figure 3.6 The average percent removal of carbazole and 3- and 4-ringed PAHs by four mixed creosote-degrading laboratory populations incubated for 4 weeks with OCT supplemented with carbazole and phenol.

was supplemented with carbazole and phenol. Phenol was not detected in any of the cultures after the 4-week incubation. Greater than 90% of each of acenaphthene, dibenzofuran, fluorene, the methyldibenzofurans, dibenzothiophene, phenanthrene, and themethylphenanthrenes was removed by the RMH Soil cultures after 4 weeks whereas the loss of these compounds ranged from 60-90% in the other cultures during this time (Fig. 3.6a). Less than 35% of the anthracene present was removed in all cultures except the RMH Soil culture in which about 60% was lost. The ESSO AG and RMH Soil cultures removed between 75% and 80% of the added carbazole, whereas only 20% and 40% were degraded in the ERN BIO and SL cultures, respectively (Fig. 3.6b). Again, biodegradation of the 4-ringed compounds was low (less than 35%) after 4 weeks of incubation, except for the ESSO AG culture in which close to 50% of the fluoranthene present was removed. Of all the cultures, the RMH Soil culture had most extensively removed OCT compounds and carbazole after 4 weeks of incubation.

After 8 weeks of incubation, more loss of the monitored compounds was evident (Fig. 3.7). In all of the extracts, none of the following were detected: acenaphthene, dibenzofuran, fluorene, the methyldibenzofurans, dibenzothiophene, and the methylphenanthrenes. Greater than 95% of the phenanthrene present was removed in all cultures. The RMH Soil culture removed 88% of the anthracene present, whereas about 65% was lost in the other cultures. For carbazole, 97% was removed by ESSO AG, 88% by RMH Soil, 71% by SL, and only 51% by ERN BIO after 8 weeks of incubation. The ESSO AG culture removed fluoranthene and pyrene to the greatest extent of all the cultures, with 75% and 54% loss, respectively. There was more loss of the 4-ringed compounds in each culture after 8 weeks than after 4 weeks of incubation. Overall, more extensive compound removal was observed for RMH Soil, ERN BIO, and SL cultures when incubated with OCT as a sole carbon source. For the ESSO AG culture, however, more extensive compound removal was seen when incubated in the presence of OCT, carbazole, and phenol.



7. Anthracene
8. Carbazole
10. Fluoranthene
11. Pyrene
12. Benzo(a)fluorene
13. Benzophenanthrene
14. Chrysene

Figure 3.7 The average percent biodegradation of carbazole and 3- and 4-ringed PAHs by four mixed creosote-degrading populations incubated for 8 weeks with OCT supplemented with carbazole and phenol.

3.3.3 Removal of OCT compounds at different incubation temperatures

Although it was evident that biodegradation or biotransformation of creosote compounds occurred when cultures were incubated at 28°C, it was unknown if the same extent of removal would occur at a lower temperature. DT Mixed and RMH Soil cultures were chosen as representative cultures to test their abilities to remove OCT compounds at 10°C, a temperature which closely approximates that of groundwater at the Borden aquifer (MacFarlane *et al.*, 1983). Sterile controls were incubated at 10°C along with the cultures. Replicate cultures and sterile controls were incubated simultaneously at 28°C to compare with 10°C results. Cultures were incubated for 4 weeks or 8 weeks, and extracted after the appropriate incubation time under acidic conditions. The resulting organic extracts were analyzed by GC.

Table 3.1 shows the percentage removal of each of 14 compounds in each culture at the two temperatures after incubation for 4 or 8 weeks, calculated from the sterile controls. Overall, there was greater removal when cultures were incubated at 28°C than at 10°C, and more loss was evident after 8 weeks than after 4 weeks. There was less of each compound remaining in the DT Mixed culture compared to the RMH Soil culture for both temperatures and incubation times. For example, in the RMH Soil culture incubated at 10°C for 4 weeks, there was almost no removal of any compound except naphthalene, the methylnaphthalenes, and biphenyl, whereas in the DT Mixed culture, some removal of all compounds except benzo(a)fluorene, benzophenanthrene, and chrysene was evident at 10°C after 4 weeks of incubation. Furthermore, none of the compounds monitored were detected by GC in the DT Mixed culture extract after 8 weeks of incubation at 28°C, whereas 4-ringed compounds persisted in the RMH Soil culture after 8 weeks.

Table 3.1 Percentage removal of selected creosote compounds in the DT Mixed and RMH Soil culture supernatants after incubation at 10°C or 28°C for 4 or 8 weeks.

COMPOUND	RMH Soil				DT Soil			
	10°C		28°C		10°C		28°C	
	4 wks	8 wks	4 wks	8 wks	4 wks	8 wks	4 wks	8 wks
Naphthalene	100	100	NA	NA	100	100	NA	NA
Methylnaphthalenes	72	87	100	NA	92	91	100	NA
Biphenyl	80	78	100	NA	88	89	100	NA
Acenaphthene	1	50	97	100	88	97	100	100
Dibenzofuran	15	75	98	100	84	90	100	100
Fluorene	0	48	95	100	66	79	98	100
Dibenzothiophene	0	61	100	100	71	85	100	100
Phenanthrene	0	59	96	100	70	81	98	100
Anthracene	0	14	70	100	4	30	77	100
Fluoranthene	0	3	58	58	3	43	70	100
Pyrene	0	0	0	26	55	80	63	100
Benzo(a)fluorene	0	0	15	21	0	19	45	100
Benzophenanthrene	0	0	0	0	0	23	43	100
Chrysene	0	3	0	0	0	20	4	100

NA=not applicable because this compound had evaporated from the sterile control

3.3.4 Mineralization of compounds in creosote

In order to determine the ability of OCT-degrading cultures to mineralize various creosote compounds, the DT Mixed culture was incubated with OCT and one of the following ^{14}C-labelled compounds: phenol, naphthalene, biphenyl, phenanthrene, anthracene, and carbazole. Figure 3.8 shows the mineralization of each of the radiolabelled compounds during a 600 h (25 day) incubation period. Naphthalene was mineralized fastest and to the greatest extent, with 67% of the radioactivity recovered as CO_2 after 3 days of incubation. The percentage $^{14}CO_2$ released for ^{14}C-carbazole also reached 67%, but not until after 5 days of incubation. Biphenyl was mineralized to a maximum of about 40% within 3 days, whereas phenanthrene mineralization reached about 40% in 3 days and reaching close to 60% by 25 days of incubation. Only about 26% of ^{14}C-phenol was mineralized by this culture.

3.3.5 Characterization of creosote-degrading populations

3.3.5.1 Degradation of aromatic hydrocarbons on agar plates

Using the spray plate method and plates containing naphthalene, phenanthrene, anthracene, or pyrene, it was found that specific aromatic hydrocarbon-degrading organisms were present in the OCT-degrading cultures. There were several naphthalene- and phenanthrene-degraders present in all of the seven cultures, although counts were not made. No clearing of plates sprayed with anthracene or pyrene was seen when the SL, ERN BIO, or ESSO AG cultures were plated, although some clearing of these substrates was seen with the DT enrichment cultures and RMH Soil. Thus, the populations did contain organisms which were capable of utilizing 2-, 3-, and 4-ringed aromatic compounds as sole carbon sources. No attempts were made to purify any of these specific hydrocarbon-degraders, although these liquid cultures could serve as useful sources of pure organisms for future degradation studies of an aromatic creosote compound.

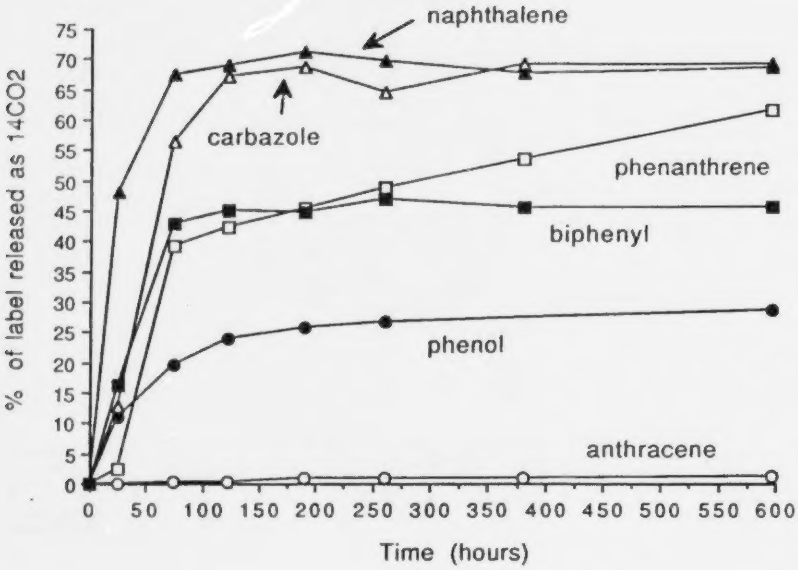


Figure 3.8 Mineralization of ^{14}C-labelled aromatic compounds added to OCT in the creosote-degrading DT Mixed culture.

3.3.5.2 Mineralization of saturated and aromatic hydrocarbons

While it was clear that the creosote-degrading populations in these studies were capable of biodegrading or biotransforming aromatic hydrocarbons, it was not known whether they were also capable of degrading saturated hydrocarbons, which are prevalent in many crude oils. SL, originally an oil-degrading population, was tested for its ability to degrade saturated hydrocarbons in PB crude oil and mineralize ^{14}C-*n*-hexadecane after having been maintained on aromatic-rich creosote for 4 years (denoted SL-OCT). An SL culture maintained only on PB crude oil (SLPB) for the same 4 years was used for comparison of degradative capabilities. As well, the DT Medium and DT Mixed cultures, never having been exposed to saturated hydrocarbons in the laboratory, were also tested for their abilities to remove and mineralize saturated hydrocarbons in PB crude oil after being incubated with creosote for 4 years. All cultures were also tested for their abilties to mineralize ^{14}C-phenanthrene and biodegrade the aromatic fraction of PB oil.

As seen in Table 3.2, greater than 45% of the label from ^{14}C-phenanthrene was recovered as $^{14}CO_2$ in all of the cultures after 7 days of incubation, and this value increased by 6 to 8% after 28 days. For the DT cultures, less than 5% mineralization of *n*-hexadecane occurred after 28 days, whereas in both SL-OCT and SLPB cultures, about 34% of the *n*-hexadecane label was recovered as $^{14}CO_2$. SL-OCT mineralized *n*-hexadecane to the greatest extent, with 43% being recovered as $^{14}CO_2$ after 28 days. Therefore, although SL had been incubating with aromatic-rich creosote as its sole carbon source for 4 years, it was still capable of utilizing a saturate-rich substrate like PB oil as a carbon source. The GC analyses of the saturate and aromatic fractions from the cultures agreed closely with the mineralization results. By day 7, the aromatic compounds detected by GC in the sterile control were not detected in the aromatic fractions from any of the cultures. Fig. 3.9 shows the chromatograms of the saturate fractions of a sterile control and the four cultures after 7 days of incubation. Within 7 days, loss of peaks was

Table 3.2 Percentage of label recovered as $^{14}CO_2$ in creosote- and oil-degrading cultures incubated with ^{14}C-*n*-hexadecane or ^{14}C-phenanthrene in PB oil for 7, 14, or 28 days.

	% $^{14}CO_2$ RECOVERED AFTER:		
SUBSTRATE & CULTURE	7 DAYS	14 DAYS	28 DAYS
***n*-Hexadecane**			
Sterile control	< Bkg[a]	< Bkg	< Bkg
SLPB	35	34	38
SL-OCT	35	37	43
DT Mixed	1	0	2
DT Medium	1	2	3
Phenanthrene			
Sterile control	< Bkg	< Bkg	< Bkg
SLPB	50	53	57
SL-OCT	54	54	62
DT Mixed	45	49	53
DT Medium	50	54	55

[a] < Bkg, % $^{14}CO_2$ recovered was less than background counts

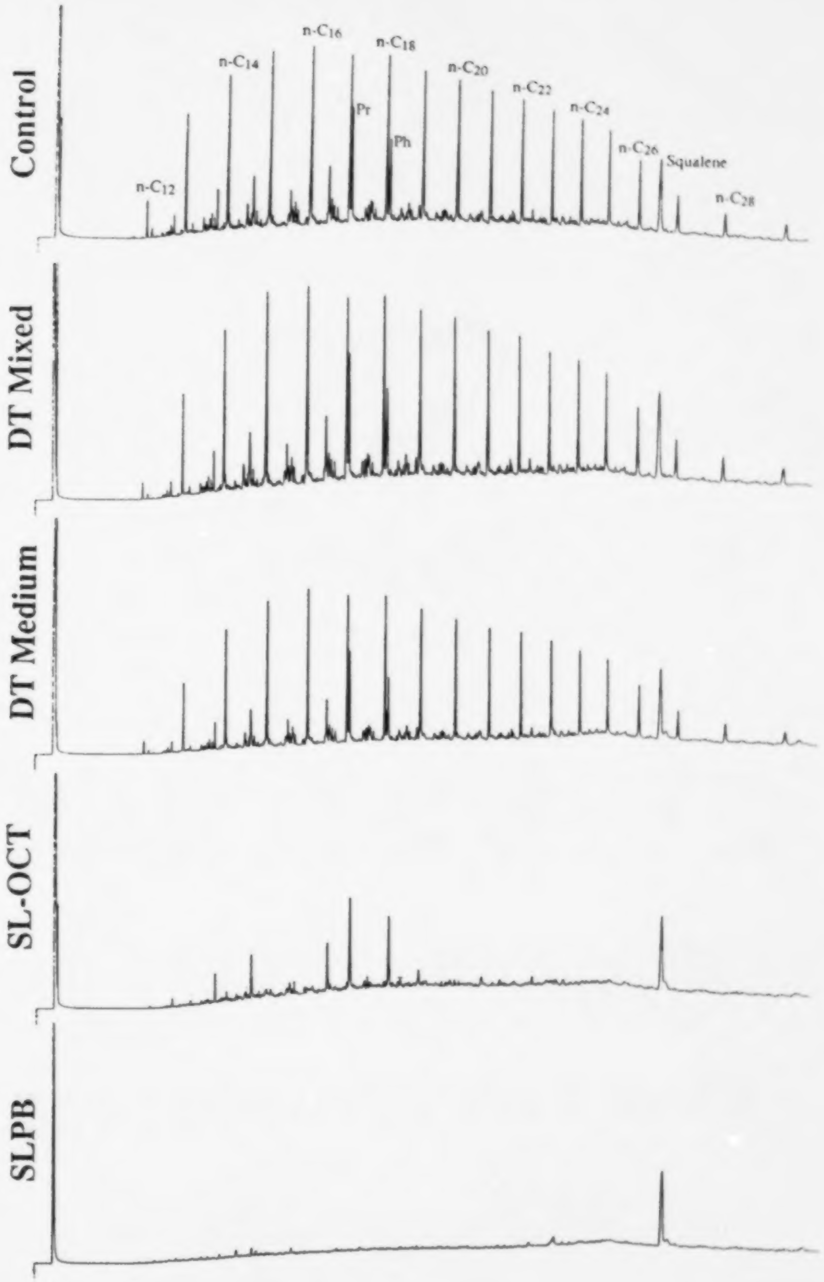


Figure 3.9 GC chromatograms showing the saturate fraction of PB crude oil in a sterile control and the different extents of removal of these compounds by four mixed cultures (Pr = pristane, Ph = phytane).

observed in the saturate fraction from SLPB, while the more recalcitrant, branched alkanes of PB crude oil, pristane and phytane, were still present in the saturate fraction from the SL-OCT culture. No loss of saturated compounds was evident in the saturate fractions from the DT Mixed and DT Medium cultures after 7 days of incubation. These saturated hydrocarbons persisted in the DT cultures, even after 28 days of incubation.

3.3.6 Anaerobic experiments

A water-soluble OCT fraction, containing phenols and some low-molecular-weight N-heterocycles and PAHs, was added to anaerobic sewage sludge incubated under methanogenic conditions and methane was monitored over time. As seen in Fig. 3.10a, the presence of water-soluble creosote compounds did not inhibit methane production in anaerobic sewage sludge incubated under methanogenic conditions. However, no increased methane production over that of the controls was seen in those cultures containing water soluble-creosote compounds. These results suggested that the water-soluble OCT compounds were not toxic to the sewage sludge population. In contrast, incubating anaerobic sewage sludge under methanogenic conditions in the presence of whole creosote inhibited methane production (Fig. 3.10b). Over the course of 98 days, methane production reached up to 11% in the controls not containing any creosote (0%), whereas methane production only rose by about 1% in those cultures containing the smallest amount of added creosote (0.8% by volume). The addition of 3.3% (by volume) or higher concentrations of whole OCT to the sewage sludge populations gave the same inhibitory results which suggested that whole OCT was toxic to some members of the sewage sludge population.

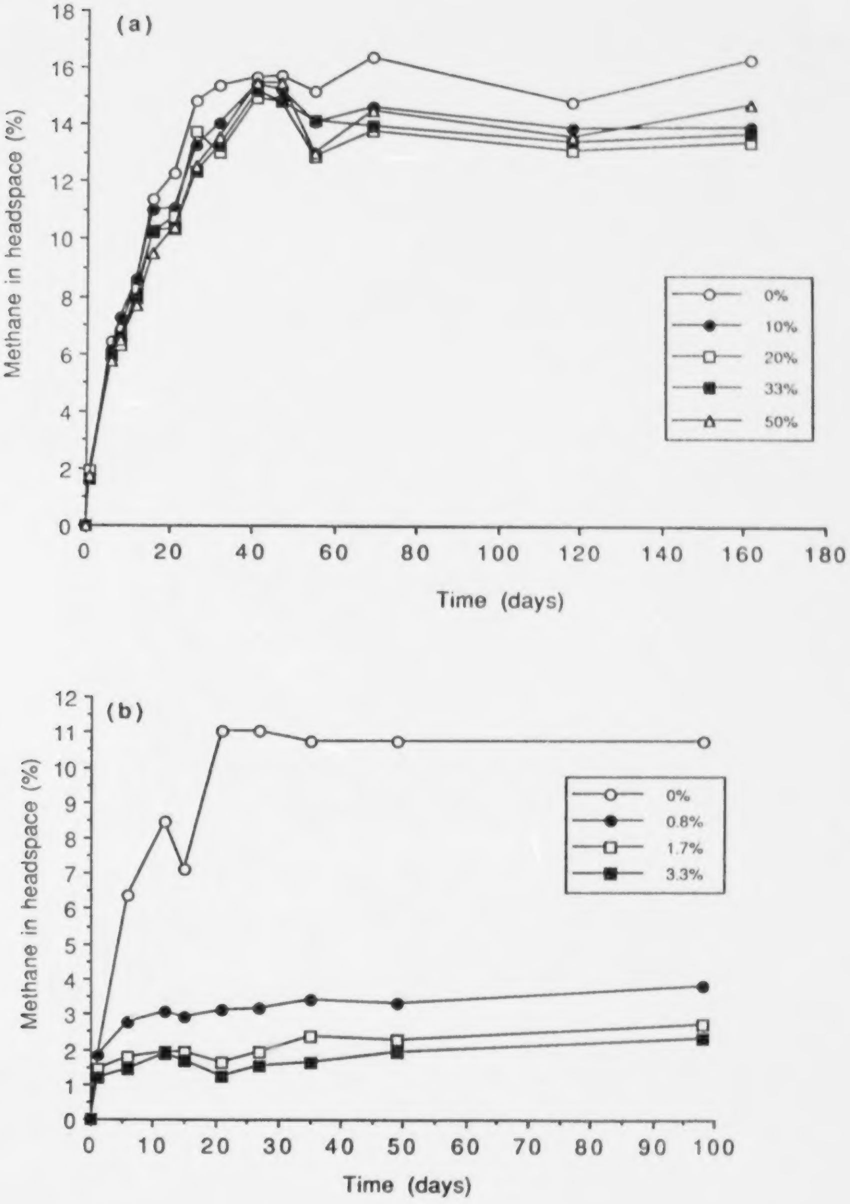


Figure 3.10 Methane production from sewage sludge incubated under methanogenic conditions with various amounts (percent by volume) of (a) water-soluble OCT compounds and (b) whole OCT.

3.4 DISCUSSION

Seven creosote-degrading cultures were established which were capable of degrading compounds in commercial coal tar creosote (OCT) under aerobic conditions. It was found that incubating these cultures with either Sidney coal tar or OCT that quinoline, isoquinoline, and indole, present in the basic extracts of culture supernatants, were removed by all of the cultures so the basic extracts were not monitored regularly. Sixteen creosote compounds which could be extracted with CH_2Cl_2 under acidic conditions were routinely monitored for their removal in each of these cultures over a period of 4 years. These included mostly 3- and 4-ringed aromatic compounds including acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)fluorene, benzophenanthrene, and chrysene, along with the methylated or dimethylated derivatives of some of these compounds. The NSO compounds monitored in the acidic extracts included dibenzofuran and dibenzothiophene, as well as carbazole when it was added. The composition of the commercial coal tar creosote used for these degradation studies has been described (Fowler *et al.*, 1994), and has been found to consist of about 83% PAHs, with phenanthrene being the predominant PAH present. The compounds present in the phenolic and polar fractions of OCT were not monitored in these studies.

Although preliminary experiments using Sidney coal tar resulted in some removal of 1- and 2-ringed compounds by the SL, ERN BIO, ESSO AG, or RMH Soil cultures, a greater extent of removal of 3- and 4-ringed compounds was observed when these cultures were incubated with OCT. Sidney coal tar, being whole coal tar and not solely a distillation fraction (like creosote), may have contained some compounds which were inhibitory to these microbial populations, although growth of these cultures was evident in the presence of this coal tar. It may also have been that the populations had acclimatized to the presence of an aromatic-rich substrate by the time they were incubated with OCT and extent of removal measurements began (about 1 year). The addition of OCT resulted in increased removal of 3- and 4-ringed PAHs in these cultures, and this was also evident in the three cultures enriched from creosote-contaminated soil. Fluorene and phenanthrene were removed by at least 85% in the all of the cultures after 4 weeks, and were no longer detected after 8 weeks of incubation (Fig. 3.5). Although anthracene has the same molecular weight as phenanthrene, this compound was more recalcitrant in the cultures. This is likely a reflection of the lower aqueous solubility of anthracene (0.07 mg/L at 25°C) than that of phenanthrene (1.3 mg/L at 25°C) (Mueller *et al.*, 1989).

Overall, the DT Medium and DT Mixed populations were the best degraders of 3- and 4-ringed compounds in creosote when incubated with OCT as a sole carbon source including fluoranthene, pyrene, benzo(a)fluorene, benzophenanthrene, and chrysene, although RMH Soil removed anthracene to the greatest extent after 8 weeks of incubation (Fig. 3.5b). When carbazole and phenol were added to ESSO AG, ERN BIO, SL, and RMH Soil cultures, the monitored compounds were most extensively removed by the RMH Soil culture after 4 weeks of incubation (Fig. 3.6a and b), although the ESSO AG culture emerged as the best carbazole-degrading culture after 8 weeks of incubation (Fig. 3.7). No metabolites were detected by the GC methods used for these experiments.

These observations show that the best degraders of 3- and 4-ringed PAHs were present in cultures enriched from creosote-contaminated soils, although long maintained laboratory oil-degrading populations incubated with OCT were able to remove many of the 3-ringed PAHs present in this creosote. These results may be explained by the fact that the DT soil cultures were enriched from soil which had been laden with creosote for a long period of time, thus the naturally occurring populations were acclimated to presence of creosote compounds and likely harboured many of the genes necessary for the degradation of aromatic compounds, especially HMW PAHs. The former oil-degrading populations, however, had been long maintained on Prudhoe Bay crude oil which is abundant in saturated hydrocarbons, but contains a lower concentration of aromatic compounds including napthalene, methylnaphthalenes, biphenyl, and phenanthrene (Foght *et al.*, 1990). Thus, these populations may not have the genetic capability to biodegrade or biotransform a mixture of compounds rich in aromatics like creosote, and were not able to remove many of the 4-ringed PAHs in OCT. The RMH soil culture, having been sampled from soil freshly contaminated with oil, was a better source of aromatic hydrocarbon-degraders than the former oil-degrading laboratory cultures, but not as good as the DT soil enrichments, likely because these microorganisms in this soil had not received longtime exposure to aromatic compounds. Numerous other studies have shown that microbial populations from contaminated environments are far better at degrading the contaminants than populations from pristine environments (Leahy and Colwell, 1990; Mueller *et al.*, 1994).

This ability of different populations to degrade different kinds of compounds was further demonstrated when cultures enriched from creosote-contaminated soil and oil-degrading laboratory populations were tested for their ability to mineralize ^{14}C-phenanthrene and ^{14}C-*n*-hexadecane in the presence of PB crude oil which is abundant in saturated hydrocarbons. Both SL maintained on PB oil and SL maintained on OCT were able to mineralize *n*-hexadecane and degrade the saturate fraction of PB oil, whereas DT Mixed and DT Medium could not (Table 3.2). Presumably, members of the DT cultures do not harbour genes for the utilization of saturated hydrocarbons. The DT cultures did mineralize ^{14}C-phenanthrene, however, and were able to biodegrade or biotransform compounds in the aromatic fraction of PB oil, which was not surprising given their long exposure to aromatic compounds.

Upon testing numerous bacterial isolates for the ability to produce $^{14}CO_2$ from ^{14}C-*n*-hexadecane and ^{14}C-phenanthrene in PB oil, Foght *et al.* (1990) found that none of the isolates was able to mineralize both compounds. The conclusion drawn from this study was that a mixed population of microorganisms with different hydrocarbon-degrading capabilites was necessary to degrade the saturated and aromatic hydrocarbons in oil. Although the DT soil cultures are mixed bacterial populations, the microorganisms were only capable of biodegrading the aromatic compounds and not the saturated hydrocarbons in PB oil.

The mineralization of some ^{14}C-labelled compounds found in creosote by the DT Mixed culture showed naphthalene being mineralized fastest and to the greatest extent when added to OCT (Fig. 3.8). Phenanthrene and biphenyl were mineralized at about the same rate, and anthracene mineralization was not observed within a 25-day incubation period. These results agree well with those observed by other researchers, as it has generally been accepted that the rates of mineralization of aromatic compounds are related to aqueous solubilities (Leahy and Colwell, 1990). The low mineralization of phenol in this culture was puzzling because phenol is more water-soluble than any other of the aromatic compounds tested and is a monoaromatic compound which is generally easier to biodegrade than 2- or 3-ringed PAHs. Although this culture did not receive prior exposure to carbazole, it was able to mineralize carbazole to the same extent as naphthalene (about 65%), thus would be a good source of carbazole-degrading bacteria.

When the RMH Soil and DT Mixed cultures were incubated at 10°C with OCT, less creosote degradation was observed than when they were shaken at 28°C which is in accordance with previous observations that rates of biodegradation generally decrease with decreasing temperature (Leahy and Colwell, 1990). The DT culture, however, degraded 70 to 100% of the 2- and 3-ringed PAHs (except anthracene) at 10°C within 4 weeks, compared to the RMH Soil culture which did not (Table 3.1). Again, this observation demonstrates the different genetic and enzymatic capabilites of the bacteria present in the different populations.

Table 3.1 shows that the DT Mixed culture removed close to 80% of the anthracene in OCT at 28°C after 4 weeks of incubation, whereas Fig. 3.8 showed that anthracene was not mineralized by this culture after 25 days of incubation. This apparent discrepancy may be explained if this culture is only capable of biotransforming anthracene (for example, to a deadend product) and not mineralizing it. If this is the case, the anthracene peak would disappear from the chromatogram but anthracene is not necessarily being degraded to CO_2 and H_2O by the DT Mixed culture.

Although many of the creosote compounds monitored in these studies were removed in the different cultures, it is not known whether a reduction in the toxicity of the creosote has occurred. Similar shake flask studies conducted to determine the biodegradation of creosote organics in contaminated groundwater showed a loss of 87 to 100% phenols, 2- and 3-ringed PAHs, and NSO compounds, and a loss of 53% of PAHs with 4 or more aromatic rings (Mueller *et al.*, 1991b). Despite the significant biodegradation of creosote compounds in these cultures, only a slight decrease in the toxicity and teratogenicity of the biotreated groundwater was observed. These toxic effects were thought to be due to the persistence of PAHs containing 4 or more aromatic rings in the cultures (Mueller *et al.*, 1991b). The persistence of HMW PAHs after biotreatment of creosote-contaminated soils and groundwaters remains a difficult challenge in the bioremediation of creosote-contaminated sites (Ellis *et al.*, 1991; Mueller *et al.*, 1994).

The experiments conducted here have shown that many of the aromatic compounds present in OCT are biodegraded or biotransformed by several different laboratory populations under ideal conditions. This does not guarantee, however, that the compounds will be biodegradable under the environmental conditions at the OCT-contaminated Borden aquifer, especially because aquifer material from the site was not used as a source of microorganisms. Biodegradation of creosote components *in situ* is site-specific and depends highly on the genetic make-up of the microbial populations naturally present in the aquifer and the presence of terminal electron acceptors that can be used by these populations, such as oxygen. Generally, when creosote contaminants reach the subsurface environment and a contaminant plume develops, the oxygen present in the groundwater is rapidly consumed by aerobic microorganisms, leaving anaerobic conditions to prevail. Thus, it is important to consider the biodegradability of contaminants under anaerobic conditions.

Although some 1- and 2-ringed aromatic compounds have been found to be biodegradable under nitrate-, sulfate-, Fe(III)-reducing, and methanogenic conditions, there have been no reports of the biodegradation of compounds containing 3 or more aromatic rings under these anaerobic conditions. The addition of whole OCT to sewage sludge incubated under methanogenic conditions showed a marked inhibition of methane production compared to controls (Fig. 3.10b) which suggested that this mixture of compounds was toxic to members of the population. In addition to 1- and 2-ringed aromatic compounds, whole OCT contains several other compounds containing greater than 3 aromatic rings, many of which have extremely low water solubilities and thus may be biologically recalcitrant under methanogenic conditions.

When the water-soluble fraction of OCT was added to sewage sludge incubated under methanogenic conditions, methane production was not inhibited compared to controls (Fig. 3.10a) which suggested that the water-soluble fraction was not toxic to members of the methanogenic consortium. However, no increased methane production was observed when the water-soluble fraction was added (up to 50% by volume). The water-soluble fraction of OCT contains phenols and LMW PAHs and NSO compounds, many of which have been shown to be biodegradable under methanogenic conditions in laboratory and field studies (Erlich et al., 1982; Godsey et al., 1983; Pereira et al., 1988; Grbic´-Galic´, 1989; Godsey et al., 1992). Thus, a lack of increased methane production by the methanogenic sewage sludge consortium incubated with these compounds may be explained in two ways: (1) the sewage sludge population did not contain organisms with the capability to break down 1- or 2-ringed aromatic compounds, or (2) the concentration of compounds added in the water-soluble fraction were not high enough to elicite enhanced methane production over that of the control. This latter explanation is most likely because the sewage sludge population used for these experiments was sampled from the same source as used for other experiments from our laboratory which demonstrated the degradation of phenols and cresols under methanogenic conditions (Fedorak and Hrudey, 1984; Londry and Fedorak, 1993).

At the creosote-contaminated Borden aquifer, some natural *in situ* biodegradation of some 1- and 2-ringed compounds in creosote is thought to have occurred because phenols have disappeared as they have migrated in the groundwater away from the creosote source (although it is not known whether they were degraded under aerobic or anaerobic conditions). Quinoline and indole have been detected at the outer edges of the contaminant plume where oxygen is still available and are likely being biodegraded under aerobic conditions by the natural microbial flora (Fowler *et al.*, 1994). Other creosote compounds, like naphthalene and carbazole, have been detected in groundwater samples removed from the anaerobic zone prevalent in the rest of the contaminant plume and a novel *in situ* biotreatment technique will be tested to remediate this zone of the contaminated aquifer (Fowler *et al.*, 1994). This technique, termed "funnel and gate", involves placing sheet piling in front of the plume with a gap in the middle (the "gate") so that groundwater can flow ("funnel") through the gate. Oxygen and nutrients will be supplied to the gate in an effort to enhance the aerobic biodegradation of the creosote compounds (Barker *et al.*, 1994; Starr and Cherry, 1994;).

Although some creosote compounds are present in the contaminated plume at the Borden site, most of the water-insoluble PAHs are still present at and in the vicinity of the creosote source. *In situ* chemical oxidation with permanganate is being proposed to remediate the creosote source, and if this does not work, the contaminated source will be excavated and incinerated, which is costly (Fowler *et al.*, 1994). Cheaper alternatives to this may be to treat the creosote-contaminated material at the source using mixed populations of known creosote-degrading microorganisms like those enriched for in these studies.

The use of specially-selected microorganisms to enhance the bioremediation of contaminated groundwater has been proven effective at a creosote-contaminated site near

Pensacola, Florida (Mueller *et al.*, 1993). Following successful employment of two-stage, seeded bioreactors at the bench- and pilot-scale levels, Middaugh *et al.* (1994) set up field-scale bioreactors to remediate creosote-contaminated groundwater at this site. The seeding of the bioreactors with 15 selected bacterial strains able to degrade LMW and HMW PAHs resulted in significant losses of these compounds and a substantial decrease in toxicity of the groundwater after biological treatment (Middaugh *et al.*, 1994). The use of engineered bioreactors to treat contaminated materials above the surface can be advantageous because physical conditions for bacterial biodegradation can be optimized. However, the use of bioreactors may not be feasible due to the nature of the contaminated material, and other researchers have proposed the use of bioaugmentation to remediate creosote-contaminated soils (Otte *et al.*, 1994). This technique involves enriching for microbial populations indigenous to the contaminated site which are capable of degrading the contaminants, and then adding these populations back to the contaminated material. This technique has been successful in both laboratory-scale (Grosser *et al.*, 1991; Otte *et al.*, 1994) and field-scale (Ellis *et al.*, 1991) biotreatments of creosote-contaminated soils.

A study which compared the ability of specially-selected microorganisms and indigenous populations to biodegrade creosote compounds suggested that it may be possible to rely solely on the enhancement of activity of indigenous microorganisms to bioremediate some sites (Mueller *et al.*, 1994). However, if an indigenous population does not have the capability to degrade creosote compounds, especially HMW compounds, then the use of non-indigenous bacteria in a treatment scheme could lend itself to effective bioremediation (Mueller *et al.*, 1994).

Thus, the creosote-degrading populations enriched for in this work, especially the DT Mixed and DT Medium cultures, could be used to enhance the biodegradation of creosote-contaminated materials.

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4. IRON REDUCTION ACTIVITY IN BORDEN AQUIFER SEDIMENTS

4.1 INTRODUCTION

Microbial iron reduction in anaerobic sedimentary environments is thought to play an important role in the biogeochemical cycling of carbon, metals, and other nutrients and has recently received increased attention as an important anaerobic process in subsurface environments (for reviews, see Lovley, 1987; Lovley, 1991; Nealson and Myers, 1992; Lovley, 1993). Although it had been long considered that iron reduction in sediments was the result of non-enzymatic processes, recent studies have suggested that much of the iron reduction in sedimentary environments is enzymatically catalyzed by microorganisms (Lovley *et al.*, 1990; Lovley *et al.*, 1991; Coleman *et al.*, 1993).

Numerous microorganisms have recently been identified which are capable of obtaining energy for growth by coupling the oxidation of organic compounds to the reduction of ferric iron. These dissimilatory iron-reducers include the newly-described *Geobacter metallireducens* (Lovley *et al.*, 1993), *Geobacter sulfurreducens* (Caccavo*et al.*, 1994), *Shewanella alga* (Caccavo *et al.*, 1995), and *Desulfuromonas palmifundus* (Coates *et al.*, 1995) as well as *Desulfuromonas acetoxidans* (Roden and Lovley, 1993) and *Shewanella putrefaciens* (Lovley *et al.*, 1989a). Indications of microbial iron reduction have been observed in diverse environments. For example, *G. metallireducens* and *S. alga* were isolated from shallow freshwater sediments on the eastern U.S. coast, *D. palmifundus* was isolated from marine sediments near San Diego, California, and *G. sulfurreducens* was isolated from a hydrocarbon-contaminated ditch near Norman, Oklahoma. Microbial iron reduction has also been found to occur in deep pristine aquifers (Lovley *et al.*, 1990; Chapelle and Lovley, 1992), shallow aquifers contaminated with crude oil (Baedecker *et al.*, 1993; Lovley *et al.*, 1994), and in a landfill-leachate polluted aquifer in Denmark (Albrechtsen and Christensen, 1994).

The finding of iron-reducing bacteria and/or iron-reducing activity in diverse sedimentary environments is not surprising because ferric iron [Fe(III)] has been found to be one of the most abundant terminal electron acceptors for organic matter decomposition in many aquatic sediments and subsurface environments (Lovley *et al.*, 1987). In sedimentary or subsurface environments, the primary terminal electron acceptors for the decomposition of organic matter are oxygen, nitrate, Mn(IV), Fe(III), sulfate, and carbon dioxide. Subsurface microbial populations will utilize that terminal electron acceptor which is most energetically favorable, with oxygen being the most favored and carbon dioxide being the least favored. When contaminants enter a subsurface environment, like a naturally aerobic shallow aquifer, oxygen often becomes depleted because of increased microbial activity and anaerobic conditions prevail. The development of redox zones usually occurs, with methanogenic and sulfate-reducing activity occurring near the contaminant source and denitrifying and aerobic zones occurring near the edge of the contaminant plume. If the aquifer sediments contain Fe(III) oxides, an iron reduction zone will develop between the sulfate-reducing and the nitrate-reducing zone (Albrechtsen and Christensen, 1994). When ferric iron is available in nitrate-depleted sediments, iron-reducing microbial populations will outcompete sulfate-reducing bacteria and methanogens in the degradation of organic matter (Lovley and Phillips, 1987a; Chapelle and Lovley, 1992).

While terminal electron acceptors like nitrate, sulfate, and oxygen are found to be associated with groundwater in an aquifer, ferric iron is associated with aquifer solids (Christensen *et al.*, 1994; Albrechtsen and Christensen, 1994). Although ferric iron can be abundant in sedimentary environments, it is not necessarily present in a form that can be utilized by iron-reducing bacteria. For example, Lovley and Phillips (1986) found that freshwater sediments of the Potomac River Estuary contained large amounts of oxalate-extractable Fe(III), but most of it was in a form which was not available for use by iron-reducing bacteria. For example, Fe(III) may have been present as mixed Fe(III)-Fe(II) minerals. These same investigators had previously shown that iron-reducing populations from sediments used Fe(III) as a terminal electron acceptor when it was present as amorphous Fe(III) oxyhydroxide but not when it was supplied in a crystalline form like hematite, goethite, or akageneite (Lovley and Phillips, 1986b). Despite this, naturally-present forms of Fe(III) in sediments have been found to serve as terminal electron acceptors in iron-reducing populations from these sediments (Lovley *et al.*, 1989b; Albrechtsen and Christensen, 1994). A recent study by Lovley *et al.* (1994) has shown that the addition of Fe(III) chelators to sediments increases the bioavailability of Fe(III), allowing the biodegradation of anaerobically recalcitrant compounds like benzene to occur under iron-reducing conditions.

Numerous organic compounds have been shown to serve as electron donors for the reduction of Fe(III) to Fe(II) by iron-reducing bacteria. These include H_2, formate, acetate, priopionate, butyrate, methanol, ethanol, glucose, and lactate as well as aromatic compounds like benzoate, *p*-hydroxybenzoate, phenol, *p*-cresol, and toluene. A new sulfate-reducing bacterium, *Desulfuromonas palmifundus*, has been isolated which can obtain energy for growth by oxidizing long chain fatty acids under iron-reducing conditions (Coates *et al.*, 1995). To date, the only pure culture of an iron-reducing bacterium which has been shown to couple the oxidation of aromatic compounds to the reduction of Fe(III) and obtain energy for growth is *G. metallireducens* (Lovley *et al.*, 1993). However, there have been some reports on degradation of aromatic compounds under iron-reducing conditions in contaminated sediments. For example, benzene and toluene were degraded in sediments from a crude oil-contaminated shallow sand and gravel aquifer under iron-reducing conditions (Baedecker *et al.*, 1993). As well, these compounds were degraded rapidly under iron-reducing conditions when a Fe(III) ligand was added to sediments removed from the iron-reducing zone of a petroleum-contaminated aquifer (Lovley *et al.*, 1994). Although iron reduction zones have been identified for these and other contaminated aquifers (Baedecker and Lindsay, 1986; Christensen *et al.*, 1994), there remains a paucity of information on the degradation of pollutant compounds under iron-reducing conditions at contaminated sites.

At an old landfill site at CFB Borden, Ontario, a 5-year field experiment was set up to examine the fate of compounds in creosote in groundwater. In 1991, commercial coal tar creosote was emplaced below the water table of the shallow, sandy aquifer located downstream of the landfill site. Field studies are ongoing to monitor the dissolution of creosote contaminants and the development of a contaminated plume in this aquifer, and some preliminary results of these studies have been reported (Fowler *et al.*, 1994; King *et al.*, 1994). Phenols have essentially disappeared, and more water-soluble creosote components like quinoline have migrated to the outer edges of the contaminant plume where oxygen is available, and are likely being degraded under aerobic conditions (Fowler *et al.*, 1994). The low concentrations of dissolved oxygen in the remainder of the contaminated plume, along with low concentrations of nitrate in this aquifer and the abundance of iron led to the proposal that microbial iron reduction may be a potential process for the degradation of creosote compounds in the anaerobic zone of this contaminated aquifer.

In order to investigate this possibility, Borden sand sampled from aerobic and anaerobic zones of the aquifer were tested in the laboratory for iron-reducing activity when incubated with various substrates, including the water-soluble fraction of creosote. Preliminary findings are presented in this chapter.

4.2 MATERIALS AND METHODS

See Appendix A for media compositions and sources of chemicals used in these experiments.

4.2.1 General anaerobic techniques

Strict anaerobic techniques were used for all experiments. O_2-free gas, 30% CO_2-balance N_2, was used during the preparation of media and substrate stock solutions, and to flush syringes used for all culture work. To ensure that this gas was O_2-free before use, it was passed through a heated copper column to scavenge trace O_2. Media for iron reduction studies were prepared aerobically, then bubbled with O_2-free gas using a canula for at least 45 min before being dispensed into serum bottles. Serum bottles (either 60 mL or 158 mL) were used for all experiments. The bottles were flushed for a time with O_2-free gas through canulae, then an appropriate amount of anaerobic medium was added using a pre-flushed pipet. Bottles were flushed for at least 2 min (60 mL bottles) or 5 min (158 mL bottles) by bubbling gas through the liquid before being capped with butyl rubber stoppers and crimped with aluminum seals. Media were autoclaved at 121°C before use.

All media and substrate additions, culture inoculations, and sampling were done aseptically using sterile syringes pre-flushed with O_2-free gas. The stoppers on the syringe flushing apparatus and all bottles used were wiped with 95% ethanol to help maintain sterility.

4.2.2 Media for iron-reducers

4.2.2.1 Description of media

Three different media were used for iron reduction studies: Fe(III) enrichment medium (Caccavo *et al.*, 1992), Fe(III) citrate medium, and Fe(III) reduction medium. Fe(III) enrichment medium was used for initial Borden sand studies, but was later replaced by the other two media which were more commonly used for iron-reduction studies (Lovley and Phillips, 1988). Fe(III) citrate medium contains ferric citrate, a soluble Fe(III) source, whereas Fe(III) reduction medium does not contain a Fe(III) source. Ferric oxyhydroxide, an insoluble Fe(III) source (synthesis described below), was added to this medium later.

4.2.2.2 Synthesis of ferric oxyhydroxide

Ferric oxyhydroxide [$Fe(OH)_3$], a poorly crystalline ferric iron source, was synthesized according to Lovley and Phillips (1986a). Briefly, a 0.4 M solution of $FeCl_3$ was neutralized with 10 N NaOH to a pH of 7, then the particulate suspension was washed with H_2O to remove excess chloride ions. The lack of chloride ions was determined by mixing a drop of the wash layer with a drop of $AgNO_3$ (85 g/L) until a white precipitate was no longer observed. The $Fe(OH)_3$ suspension (in H_2O) was bubbled with O_2-free gas prior to addition to serum bottles containing Fe(III) reduction medium using a sterile, pre-flushed syringe. Generally, 50 mL of Fe(III) citrate medium was added to 158-mL serum bottles, whereas 10 mL was added to 60-mL serum bottles. If $Fe(OH)_3$ was to be added, 36 mL of Fe(III) reduction medium and 14 mL of the $Fe(OH)_3$ suspension was added to 158-mL serum bottles, and 8 mL of this medium plus 2 mL of the $Fe(OH)_3$ suspension was added to 60-mL serum bottles. This resulted in approximately 100 mmol $Fe(OH)_3$ per litre of medium.

4.2.2.3 Iron chelators

Nitrilotriacetic acid (NTA) is a ferric iron chelator which has been shown to make Fe(III) naturally present in sediments more available for microbial use and biodegradation of compounds under iron-reducing conditions (Lovley *et al.*, 1994). In some experiments described here, NTA was added at a concentration of 2 mmol/kg sediment (from Lovley *et al.*, 1994) to Borden sediments not amended with $Fe(OH)_3$. Fe(III) chelated to NTA was added to some Borden sediments, also at a concentration of 2 mmol/kg sediment. NTA-Fe(III) was synthesized by combining equimolar concentrations of NTA and $FeCl_3$ (Lovley *et al.*, 1994). These solutions were prepared using O_2-free H_2O and were autoclaved before use.

4.2.3 Substrates

4.2.3.1 Aromatic and non-aromatic compounds

These substrates were generally prepared as stock solutions by boiling H_2O, cooling while bubbling with O_2-free gas, then dispensing an appropriate volume of H_2O into serum bottles containing preweighed substrate. Substrate stock solutions were autoclaved before use and stored at 4°C. These solutions were generally prepared such that the addition of 1 mL to a culture bottle resulted in the appropriate substrate concentration. The following substrates were prepared as stock solutions and added at the following concentrations for iron reduction experiments: sodium acetate (10-50 mM), glucose, sodium lactate, butyrate, propionate (all at 10-20 mM), phenol, *p*-cresol, sodium benzoate, and *p*-hydroxybenzoate (all at 200 mg/L). Benzene and toluene (200 mg/L) were added directly to serum bottles capped with Teflon-lined stoppers using a glass syringe.

4.2.3.2 Water-soluble creosote compounds

Water-soluble compounds present in the coal tar creosote (OCT) used for the creosote experiments at the Borden aquifer site in Borden, Ontario were added to Borden core material as carbon sources to test for iron-reduction activity. The composition of this creosote has been reported (Fowler *et al.*, 1994). To prepare the water-soluble fraction (also referred to as the aqueous fraction), 1 part OCT was shaken with 40 parts sterile H_2O for 24 h. Water phase (yellow) was aseptically transferred to a sterile serum bottle, carefully avoiding the darkly-colored creosote phase, and was bubbled with O_2-free gas before being capped and sealed. A sample of the water phase (20 mL) was extracted with dichloromethane under both acidic and basic conditions, and the resulting organic extracts were analyzed by GC to observe and tentatively identify the water-soluble compounds in OCT. For the experiments described here, 1.0 mL of the anaerobically-prepared aqueous OCT solution was added to 60-mL serum bottles containing 10 mL medium and $Fe(OH)_3$ and 1.0 mL inoculum, thus the cultures contained about 8% (by volume) of the aqueous OCT solution. The concentration of the individual water-soluble compounds added to iron-reducing cultures was not determined in these studies.

4.2.4 Organisms and inocula used for iron reduction studies

4.2.4.1 *Geobacter metallireducens*

G. metallireducens, a well-characterized iron-reducing bacterium, was obtained from the American Type Culture Collection (ATCC # 53774). This organism was used as a model to verify the culture and assay methods being used for iron reduction studies with Borden sand and was added to Borden sediments in some experiments. *G. metallireducens* was maintained under strict anaerobic conditions by regular transfers in Fe(III) citrate medium with 50 mM acetate as a carbon source (Lovley *et al.*, 1993) and incubated at 35°C.

4.2.4.2 Borden aquifer material

4.2.4.2.1 Sources of cores and amount of Fe(III)

Three different core samples were obtained from the shallow, sandy aquifer experimental site in Borden, Ontario (sampled by scientists from the University of Waterloo): two were aerobic cores sampled from a relatively clean area (sampled November, 1992 and September, 1993), and one was from the anaerobic zone within the OCT-contaminated plume (sampled October, 1994). Core samples were shipped to the university of Alberta and stored at 4°C. Analyses were carried out on each of these core samples to determine the amount of microbially-reducible Fe(III) present using the hydoxylamine-hydrochloride assay (described in Section 4.2.5.2). The average amount of microbially-reducible Fe(III) per gram of sandy material in aerobic core #1 was 130 µg/g wet sediment, that of aerobic core #2 ranged from 50 µg/g to 200 µg/g, and that of the anaerobic core averaged 200 µg/g.

4.2.4.2.2 Culturing of Borden core material

Sand sampled from an aerobic zone of the Borden aquifer was weighed aseptically in air and dispensed into sterile serum bottles which were then flushed with O_2-free gas for at least 5 min before being capped and sealed. Anaerobic medium and substrate solutions were then added using syringes.

Borden sand sampled from an anaerobic zone was weighed into sterile serum bottles in an anaerobic glove box containing a gas mixture of 10% H_2, 5% CO_2, and 85% N_2. Bottles were placed in the glove box at least 5 days before they were to be used to allow them to become filled with anaerobic gas. The bottles were capped and sealed before being removed from the anaerobic glove box. Media and substrate solutions were prepared separately and added to the sand-containing bottles using syringes. Sterile controls were prepared by autoclaving weighed-out sand three separate times for a total of 60 min. All Borden sand cultures were incubated in the dark at room temperature (22-24°C) unless otherwise noted. When Borden sand cultures were transferred (1.0 mL, 8% by volume), medium and sediment were mixed before removal of culture supernatant using a sterile, pre-flushed syringe.

For most experiments, only one bottle of aquifer material was set up for each substrate tested because of the limited amount of core material that was available. Thus, statistical analyses were not performed for the data obtained.

4.2.5 Iron assays

4.2.5.1 Measurement of Fe(II) production

Iron reduction activity was measured by monitoring the increase in Fe(II) over time in cultures and controls incubated with or without the addition of Fe(III). The presence of Fe(II) was measured using the ferrozine assay, as outlined in Lovley and Phillips (1986a). Ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,3,5-triazine] reacts with divalent cations to form a stable magenta complex which is water-soluble and can be measured spectrophotometrically (Stookey, 1970). Briefly, 0.1 mL of sample was removed from each serum bottle using a syringe and added to a test tube containing 5 mL of 0.5 M HCl. The tubes were vortexed and allowed to sit for at least 15 min. Then, 0.1 mL of the acidified sample solution was added to a separate test tube containing 5 mL of ferrozine (1 g/L) in 50 mM HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid) buffer (at pH=7). The tubes were vortexed again and A_{562} was determined on a Philips Model PU 8740 UV/Vis scanning spectrophotometer. Fe(II) standards were prepared using ferrous ammonium sulfate (described in Appendix C) to convert the absorbance readings into μg values. The data for Fe(II) production are reported as μg Fe(II) per 0.1 mL of culture supernatant sampled.

4.2.5.2 Measurement of microbially-reducible Fe(III)

Lovley and Phillips (1987b) devised a method of determining microbially reducible ferric iron using hydroxylamine-HCl. This method was applied to the Borden aquifer sediments used in these experiments. Six 0.1 g samples of each core sample were weighed into test tubes. For three of these, HCl-extractable Fe(II) was measured using the procedure in Section 4.2.5.1. For the other three samples, 5 mL of 0.25 M hydroxylamine-HCl, which reduces Fe(III) to Fe(II), was used instead of HCl as the extractant (Lovley and Phillips, 1987b). Fe(II) was then measured as above using the ferrozine assay. The amount of hydroxylamine-reducible Fe(III) was calculated as the difference between the Fe(II) present in the hydroxylamine-HCl extract and that present in the HCl extract.

4.2.6 Substrate analyses

For some experiments, the substrates which were amenable to analysis by GC or high performance liquid chromatography (HPLC) were monitored. These included acetate, propionate, butyrate, phenol, *p*-cresol, benzoate, and *p*-hydroxybenzoate. Samples for substrate analysis were removed (0.4 mL) and centrifuged to remove particulate matter (eg. sand, $Fe(OH)_3$). The supernatants were then analyzed by one of the methods described below.

4.2.6.1 Acetate, propionate, and butyrate analysis

The concentrations of acetate, propionate, and butyrate were measured by GC using a HP model 5700 gas chromatograph equipped with a packed glass column (2 m X 2 mm). The packing material consisted of GP 10% SP 1000/1% H_3PO_4 on 100/200 chromosorb WAW (Supelco). The oven temp was 120°C, the injector port was at 225°C and the flame ionization detector was at 250°C. A HP Model 3390A integrator was used to determine peak areas. Standards of acetate (5-30 mM), propionate (2.5-20 mM), and butyrate (2.5-20 mM) were injected on the GC to prepare standard curves prior to analyzing culture samples in order to determine substrate concentrations in the samples. Ten microlitres of 4 M H_3PO_4 was added to 90 µL of standards or culture supernatants prior to GC analysis.

4.2.6.2 Aromatic substrate analysis

The concentrations of phenol, *p*-cresol, benzoate, and *p*-hydroxybenzoate were measured on a HP series 1050 HPLC using a reversed-phase C_{18} column (Lichrospher 100 RP-18, 125 X 4 mm column with 5 µm diameter packing material, HP). The mobile phase was 1% aqueous acetic acid and methanol (60:40) and compound peaks were detected using a wavelength of 254 nm. A HP integrator was used to determine peak areas of the substrates.

4.2.6.3 Aqueous OCT composition

To detect and tentatively identify water-soluble creosote compounds, concentrated CH_2Cl_2 extracts of the water-soluble OCT fraction were analyzed on a HP model 5390 A gas chromatograph equipped with a FID. A DB-5 column was used with the following temperature program: 90°C for 2 min, increasing by 4°C per min, to 250°C, held for 4 min. The injector and detector temperatures were at 250°C.

4.3 RESULTS

4.3.1 Experiments with aerobic core samples

4.3.1.1 Aerobic core #1

Borden aquifer material sampled from an aerobic zone in November, 1992 was incubated anaerobically in serum bottles containing Fe(III) enrichment medium in the presence or absence of added $Fe(OH)_3$ and one of the following substrates: glucose, lactate, benzoate, *p*-hydroxybenzoic acid (all at 10 mM), phenol, and *p*-cresol (at 200 mg/L). Sterile controls were set up, as well as substrate-free controls which received O_2-free H_2O in place of a carbon source. Fe(II) production was monitored weekly. By 3 weeks of incubation, the $Fe(OH)_3$ layer had undergone a color change from rusty-colored to gray or brown in all of the bottles containing this added iron source and the orange color of the Fe(III) enrichment medium became clear. In cultures without added $Fe(OH)_3$, the medium turned clear as well. No changes were evident in the sterile controls, although the substrate-free controls did show these color changes. From these visual observations, it appeared as though iron reduction was occurring, although the ferrozine analyses did not show a large increase in Fe(II) production, even after 7 weeks of incubation. For these early analyses, culture supernatants were sampled without prior mixing with the Borden sediments which likely accounted for the low values observed for Fe(II) production. When the supernatants were mixed with the sediments before the 8 week sampling, concentrations close to 1 mg Fe(II)/mL of culture supernatant were detected when $Fe(OH)_3$ was added, except for the sterile control which contained only about 35 μg Fe(II)/mL. For cultures containing only the Fe(III) enrichment medium which contains a soluble Fe(III) source, only slightly higher Fe(II) values were detected when the sediments were mixed with the supernatants. For example, when lactate was added as an electron donor, 27 μg Fe(II)/mL was measured when the supernatant was sampled without mixing, whereas 42 μg Fe(II)/mL was measured when the supernatant was mixed with the sediment prior to sampling.

A second experiment was set up in the same manner, but supernatants were mixed with the sandy material before sampling from the start of the experiment. The results of the Fe(II) assay showed that an increase in Fe(II) production corresponded with the color changes of both the medium and the $Fe(OH)_3$ layer. Mixing of supernatants with sediment before sampling for Fe(II) analysis was done for all future experiments. These preliminary experiments suggested that Borden aquifer sediments contained populations which were capable of reducing Fe(III) to Fe(II) and that this process was microbially mediated because Fe(II) accumulation was not observed in sterile controls.

Portions of the phenol- and glucose-containing cultures (as representative aromatic and non-aromatic substrates, respectively) were transferred to fresh medium and substrate and again, high amounts of Fe(II) were measured within 14 days of incubation (about 0.8 mg Fe(II)/mL culture) and color changes were evident compared to a sterile control. In a subsequent transfer (second transfer), only the phenol-containing culture showed a large increase in Fe(II) production, although loss of phenol was not monitored. The transferred glucose-enriched culture did not show an accumulation of Fe(II), even after 92 days of incubation. These results suggested that the transferred Borden sand populations were utilizing phenol as a growth substrate under iron-reducing conditions, but not glucose.

4.3.1.2 Aerobic core #2

Experiments with Borden aquifer material sampled from a different aerobic zone in September 1993 used a new medium for studying iron reduction activity. The Fe(III) enrichment medium (Caccavo *et al.*, 1992) that was used for the experiments described in Section 4.3.1.1 was lacking in nutrients such as Mg^{2+} and Ca^{2+} and contained a soluble Fe(III) source. For the experiments with aerobic core #2, it was desirable to set up some Borden sand cultures versus various substrates without any added iron source. Thus the aquifer material served as the Fe(III) source. Thus, the new medium used for this and all future experiments was Fe(III) reduction medium, which did not contain any soluble Fe(III) and contained numerous vitamins and minerals (Lovley and Phillips, 1988).

The new aerobic core material was initially tested for iron reduction activity versus simple substrates in the presence and absence of $Fe(OH)_3$. These included glucose, lactate, propionate, butyrate (all at 10 mM), and acetate (20 mM). Fe(II) production was monitored over time. No increase in Fe(II) production was observed when no $Fe(OH)_3$ was added to the cultures after 82 days of incubation, and the results of the cultures incubated with added $Fe(OH)_3$ are shown in Fig. 4.1. Fe(II) was produced most quickly and to the greatest extent in the lactate-containing culture, reaching up to 790 μg/0.1 mL culture after 82 days. An increase in the amount of Fe(II) was observed in the glucose-containing culture between 22 and 32 days, and in the butyrate-containing culture after 47 days. The $Fe(OH)_3$ layer changed from rusty-colored to tan-colored for the lactate and butyrate cultures and to gray for the glucose-enriched culture. No increase in Fe(II) production or color changes were observed with the addition of other substrates or in the controls.

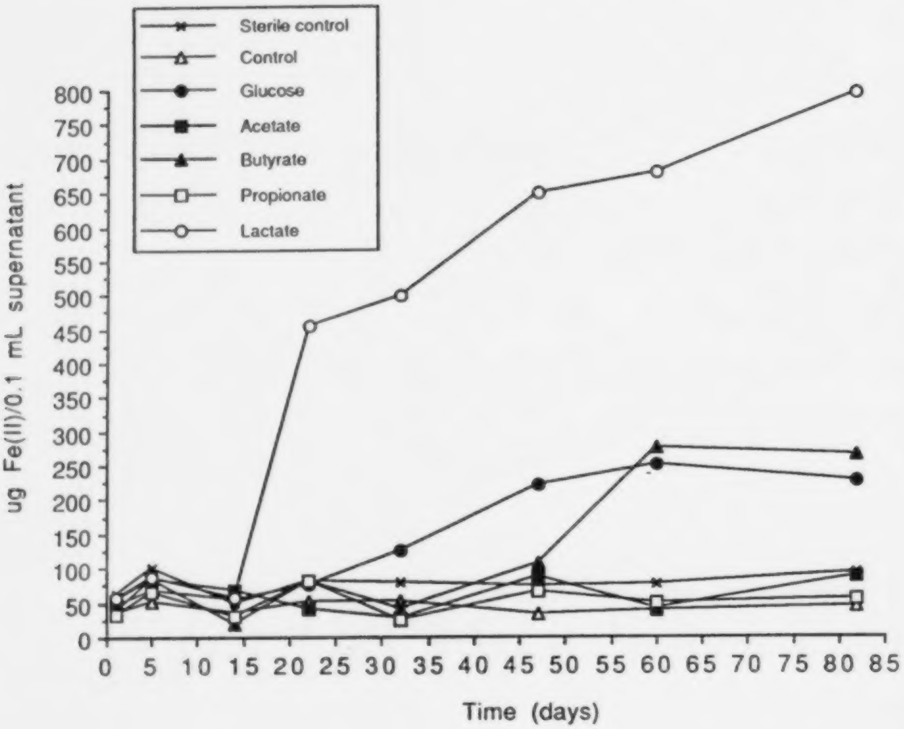

Figure 4.1 Fe(II) production in Borden sand cultures (aerobic core #2) incubated with glucose, lactate, acetate, propionate, or butyrate and $Fe(OH)_3$.

Because the lactate-, glucose-, and butyrate-containing cultures showed an increase in Fe(II) concentration, they were transferred into fresh medium and substrate containing either $Fe(OH)_3$, or soluble Fe(III) citrate (Fe(III) citrate medium was used). After 20 days of incubation, only the lactate-containing culture to which $Fe(OH)_3$ was added showed an increase in Fe(II) concentration (up to 510 μg Fe(II)/0.1 mL culture supernatant). After 150 days, the Fe(II) concentration had increased from 130 μg to 380 μg Fe(II)/0.1 mL culture supernatant in the lactate-containing culture using Fe(III) citrate as the electron donor. No increase in the amount of Fe(II) was evident for the other transfer cultures.

This aerobic aquifer material was also tested for its ability to utilize aromatic substrates under iron-reducing conditions. Benzoate, *p*-hydroxybenzoate, phenol, *p*-cresol, toluene, or benzene was added as a substrate to Borden sand in the presence or absence of $Fe(OH)_3$. After 54 days of incubation, virtually no increase in Fe(II) production was detected in any of the cultures. Even after incubating for longer than a year, no Fe(II) was produced, and no color change of the $Fe(OH)_3$ layer was evident in any one of the cultures.

A final experiment was set up with aerobic core #2. This sandy material was incubated with one of 11 different substrates under three different conditions: $+Fe(OH)_3$, $-Fe(OH)_3$, or $+Fe(OH)_3$ and *G. metallireducens*, a known Fe(III)-reducing bacterium. The substrates tested were: glucose, lactate, acetate, propionate, butyrate, phenol, *p*-cresol, benzoate, *p*-hydroxybenzoate, benzene, and toluene. Sterile controls and substrate-free controls were also set up, and the cultures were incubated at 35°C, the ideal incubation temperature for *G. metallireducens* (Lovley *et al.*, 1993). Both Fe(II) production and substrate loss were measured over time. After 98 days of incubation, all of the *G. metallireducens*-containing cultures showed an increase in Fe(II) production but only to about the same level as that observed in the control. The one exception was the toluene-containing culture in which the Fe(II) concentration of 350 μg Fe(II)/0.1 mL culture supernatant was twice as high as that of the control after 98 days. In the *G.*

metallireducens-containing cultures, the rusty-colored $Fe(OH)_3$ layers became dark brown, except for the glucose-containing culture in which the layer turned grayish-white. The only culture showing an increase in Fe(II) production over time that did not contain *G. metallireducens* was the glucose-containing culture to which $Fe(OH)_3$ was added, reaching up to 170 µg Fe(II)/0.1 mL culture after 98 days of incubation. The $Fe(OH)_3$ layer became pale gray, almost white. Although an increase in Fe(II) was seen in the cultures amended with *G. metallireducens*, virtually no decreases in the concentrations of acetate, propionate, butyrate, phenol, *p*-cresol, benzoate, and *p*-hydroxybenzoate were detected within 98 days of incubation.

4.3.2 Experiments with anaerobic core material

4.3.2.1 Experiments with non-aromatic and aromatic substrates

Aquifer material removed from the anaerobic zone between 2 and 3 metres in front of the emplaced creosote source at the Borden site was tested for iron-reducing activity. Sand was weighed into serum bottles containing Fe(III) reduction medium, with and without $Fe(OH)_3$, and with one of the following substrates: glucose, lactate, acetate, propionate, butyrate, phenol, *p*-cresol, benzoate, *p*-hydroxybenzoate, benzene, or toluene. Sterile controls and substrate-free live controls to which O_2-free H_2O was added in place of substrate were also set up. Fe(II) production was monitored over time.

Little increase in Fe(II) was seen in cultures to which no $Fe(OH)_3$ was added. Compared to the controls, the amount of Fe(II) produced in these unamended cultures ranged from 6 to 18 µg Fe(II)/0.1 mL culture supernatant for the non-aromatic substrates and from 4 to 8 µg Fe(II)/0.1 mL culture supernatant for the aromatic substrates. Some of cultures containing added $Fe(OH)_3$ accumulated significantly more Fe(II) over time. Figure 4.2a shows large increases in Fe(II) production during 217 days of incubation in cultures containing glucose, lactate, acetate, propionate, or butyrate, compared with

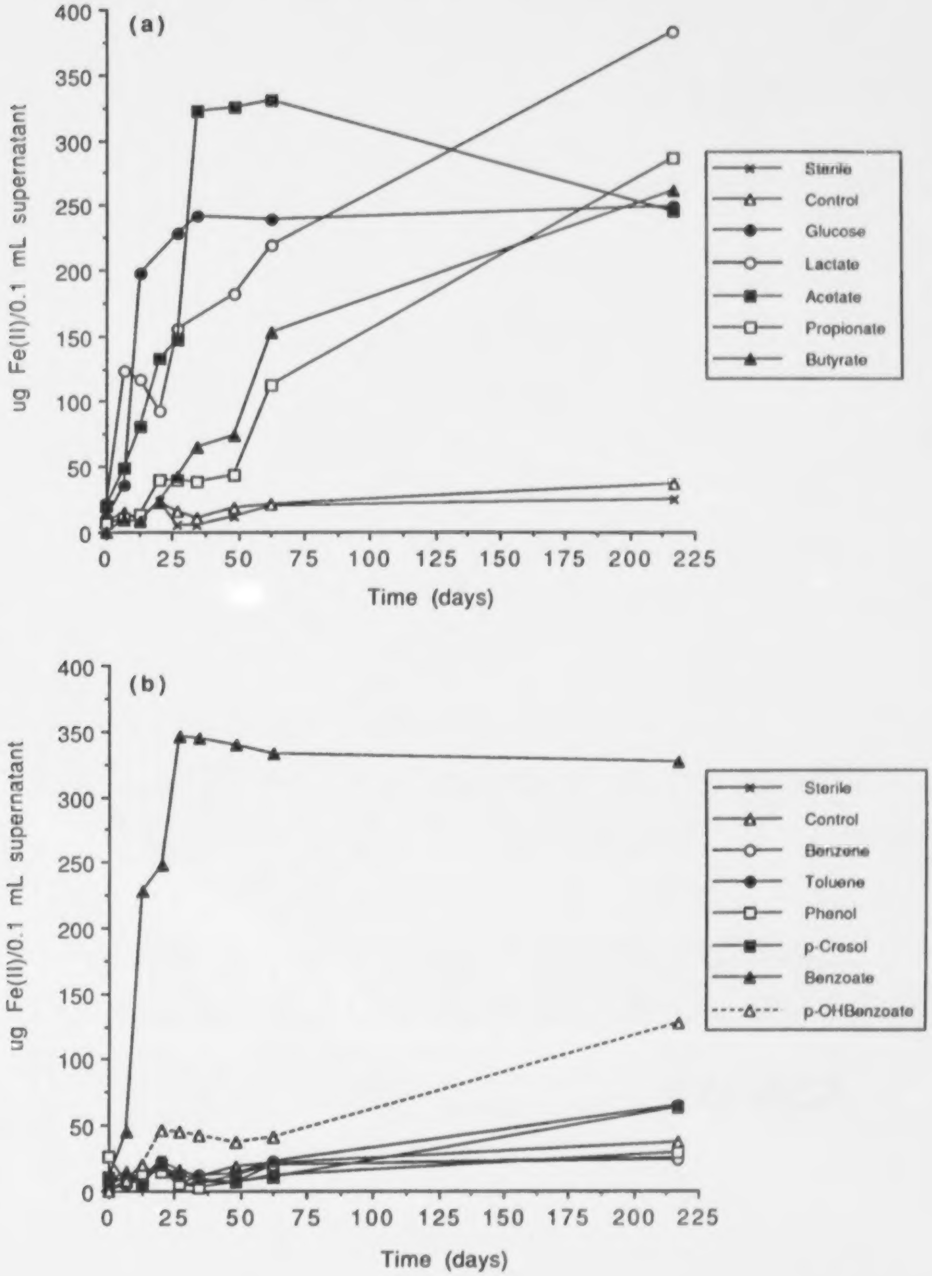

Figure 4.2 Fe(II) production in Borden sand cultures (sampled from the anaerobic zone) incubated with $Fe(OH)_3$ and (a) non-aromatic compounds or (b) aromatic compounds.

controls. Overall, lesser increases in Fe(II) production were seen when the Borden sand was incubated with aromatic substrates, except for the benzoate-containing culture which accumulated more Fe(II) than any other culture by 27 days of incubation (Fig. 4.2b).

After 14 days of incubation, the glucose-, lactate-, acetate-, and benzoate-containing cultures were transferred to fresh medium containing $Fe(OH)_3$ and the appropriate substrate, and Fe(II) production was monitored. Large accumulations of Fe(II) were observed. A subsequent transfer of these cultures again showed the same results, but after the third transfer, high Fe(II) values were detected only for the benzoate-enriched culture. This benzoate enrichment was then used as inoculum to see if Fe(II) would increase when phenol, *p*-cresol, or *p*-hydroxybenzoate were added as substrates and when Fe(III) was supplied as either insoluble $Fe(OH)_3$ or as soluble Fe(III) citrate. Cultures amended with benzoate were used as positive controls and inoculated substrate-free controls were also established. No increase in Fe(II) was observed in cultures containing any of the substrates when Fe(III) citrate was the electron donor, and the Fe(II) concentration increased only in the benzoate-amended culture containing $Fe(OH)_3$, from 70 μg/0.1 mL at the time of transfer up to 350 μg/0.1 mL culture supernatant after 20 days of incubation. No increases in Fe(II) were observed in the substrate-free controls.

Because iron-reducing activity was observed when anaerobic Borden aquifer sediments were incubated with acetate, glucose, lactate, or benzoate, new anaerobic aquifer material was again incubated with these substrates to obtain enrichments for water-soluble OCT experiments (described in Section 4.3.2.2) and to see if iron-reducing activity could be detected when NTA was added. For all previous experiments, only small increases in Fe(II) production were observed for Borden sediment cultures containing only natural iron forms (ie. when no $Fe(OH)_3$ was added). Experiments by Lovley *et al.* (1994) have shown that the addition of the Fe(III) ligand NTA made Fe(III) from oxides naturally-present in sediments more available for the degradation of organic compounds by sedimentary microorganisms. Thus, for this experiment, NTA was added to Borden sand containing one of the four substrates and no added $Fe(OH)_3$, and cultures to which $Fe(OH)_3$ was added were also established. Fe(III) chelated to NTA was also added to a separate bottle containing benzoate as a substrate. Monitoring of Fe(II) over time showed that Fe(II) accumulated only in those cultures amended with $Fe(OH)_3$ (Fig. 4.3a). However, replotting the data from the NTA-containing cultures on a smaller scale showed that there was a small amount of Fe(II) being produced in these cultures during the first 20 days of incubation (Fig. 4.3b).

The cultures above that showed increased iron reduction in the presence of $Fe(OH)_3$ were transferred to fresh medium and substrate after 28 days of incubation and within a month, the concentration of Fe(II) that had accumulated in the cultures surpassed that of the original Borden sand cultures (Fig. 4.4). After a subsequent transfer (indicated by the arrow at 57 days in Fig. 4.4), the Fe(II) values were lower for glucose, benzoate, and lactate cultures, whereas those for acetate were as high as the first transfer. After the third transfer of these cultures, the concentration of Fe(II) remained low for the benzoate and lactate cultures, were lower for the glucose transfers, but remained high for the acetate-enriched culture (Fig. 4.4). Both the glucose- and acetate-enriched cultures became tan-colored after the fourth transfer, whereas the benzoate- and lactate-enriched cultures turned dark brown. These results are in contrast to the previous transfer experiment described above in which only the benzoate-enriched culture showed continued iron-reduction activity with repeated transfers.

4.3.2.2 Experiments with water-soluble OCT components

The acid extract of the water-soluble fraction of OCT was found to be enriched in phenols including phenol, 2- and 4-methylphenol, and dimethylphenols, as well as some polycyclic aromatic hydrocarbons like naphthalene, 1- and 2-methylnaphthalene, fluorene, and phenanthrene. Compounds identified in the basic extract were quinoline, isoquinoline, and indole. Thus, the Borden sediments and transfer cultures were tested for iron-reducing

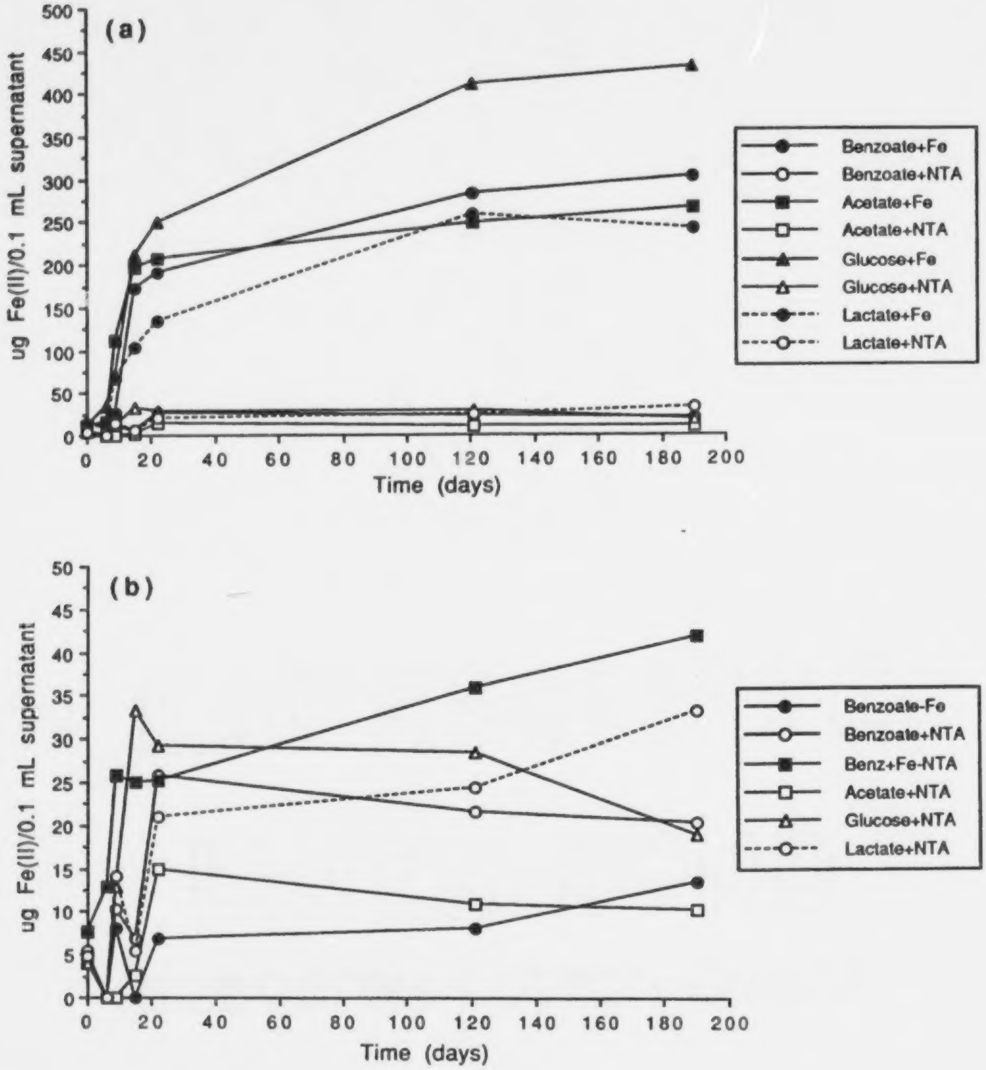

Figure 4.3 Fe(II) production in anaerobic Borden sand cultures incubated with glucose, acetate, lactate, or benzoate in the presence of $Fe(OH)_3$ or NTA (a). Data from the NTA-containing cultures were replotted on a smaller scale in (b).

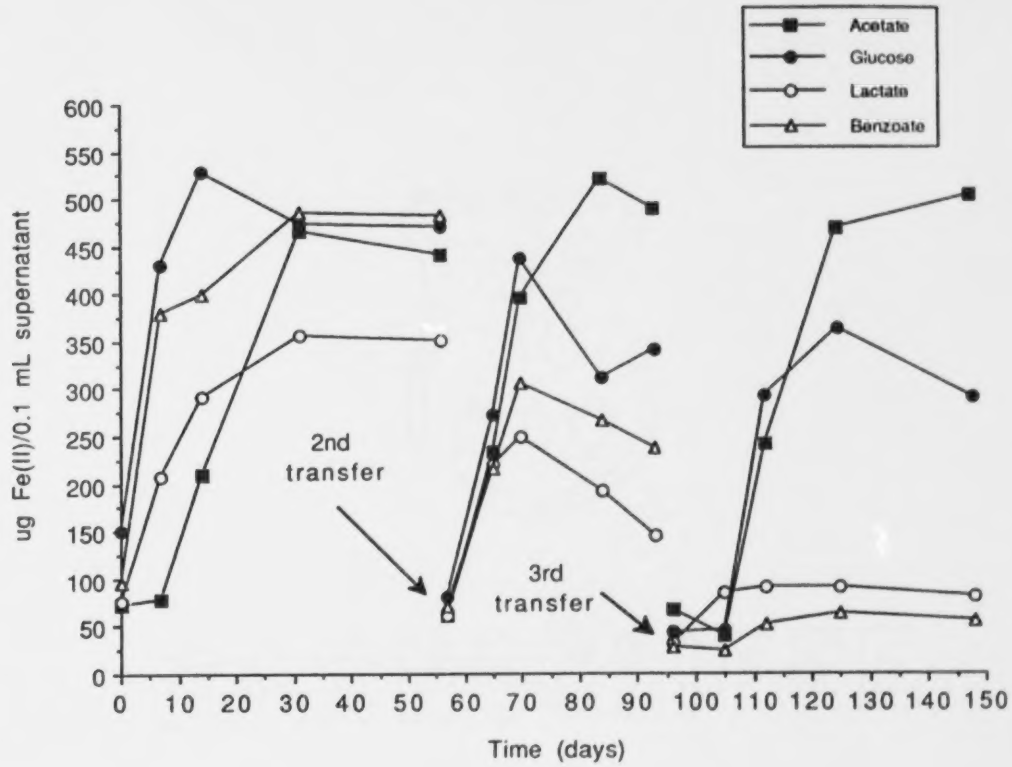

Figure 4.4 Fe(II) production in Borden sand cultures (from Fig. 4.3a) after successive transfers into medium containing glucose, acetate, lactate, or benzoate and $Fe(OH)_3$.

activity in the presence of a mixture of aromatic compounds.

Cultures enriched from Borden aquifer material sampled from the anaerobic zone were tested for their ability to reduce Fe(III) when a solution of water-soluble OCT compounds was added as an electron donor. Cultures showing increased Fe(II) production from the first experiment with anaerobic core material were initially tested including the glucose-, lactate-, acetate-, propionate-, butyrate-, and benzoate-enriched cultures (cultures from Fig. 4.2). These cultures had been incubating for 7 months before being transferred to fresh Fe(III) reduction medium amended with $Fe(OH)_3$ and aqueous OCT compounds. Figure 4.5 shows an increase in Fe(II) production in cultures originally enriched on glucose, acetate, butyrate, lactate, or propionate during 89 days of incubation in the presence of aqueous OCT compounds. Fe(II) increased in the lactate-enriched culture without a lag, whereas Fe(II) production did not increase in the butyrate-enriched culture until after 15 days, in the acetate-enriched culture until after 28 days, and in the propionate-enriched culture until after 52 days of incubation. No iron-reducing activity was seen in the glucose- or benzoate-enriched cultures after 89 days of incubation.

Transfer cultures which had been incubating in the presence of benzoate, glucose, lactate, or acetate for about 2 months (cultures from Fig. 4.4) were transferred to fresh Fe(III) reduction medium containing added $Fe(OH)_3$ and water-soluble OCT compounds. A control was also set up which did not contain inoculum but did contain the added OCT compounds and $Fe(OH)_3$. Figure 4.6 shows the accumulation of Fe(II) in the presence of aqueous OCT compounds in glucose-, acetate-, and lactate-enriched cultures over a 96-day incubation time. Virtually no increase was observed in the benzoate-enriched culture or in the control. This experiment was repeated, and similar results were observed.

Water-soluble OCT compounds were also added to anaerobic Borden aquifer material in the presence and absence of $Fe(OH)_3$. Sterile controls and controls containing O_2-free H_2O were set up to account for background Fe(II) production. Sediment cultures to which NTA or Fe(III)-NTA were also established. The increases in Fe(II) production in

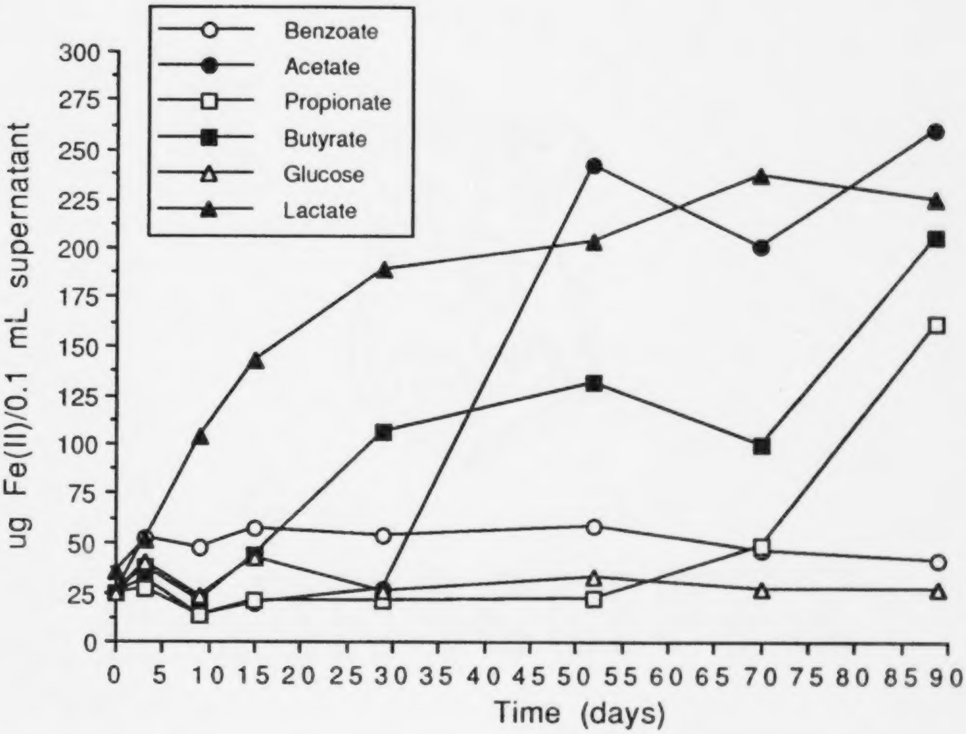

Figure 4.5 Fe(II) production in Borden sand transfer cultures incubated with the water-soluble fraction of OCT and $Fe(OH)_3$. The legend denotes the substrate on which the culture had been previously enriched (cultures from Fig. 4.2).

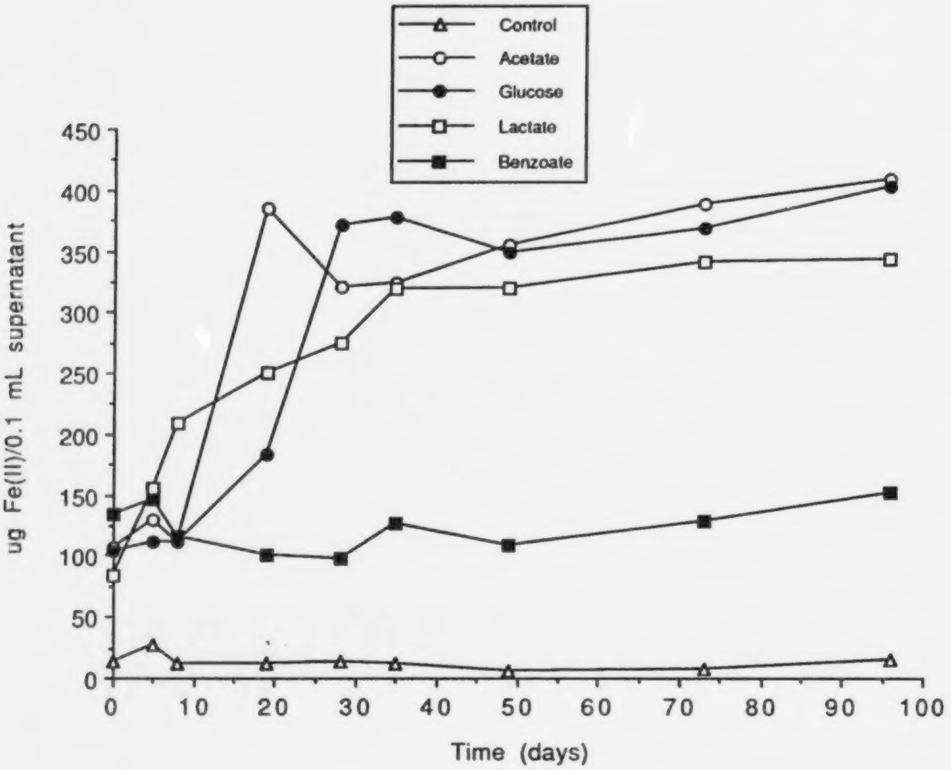

Figure 4.6 Fe(II) production in Borden sand transfer cultures, incubated with water-soluble creosote compounds and $Fe(OH)_3$. These cultures were previously enriched on glucose, acetate, lactate, or benzoate (cultures from Fig. 4.4).

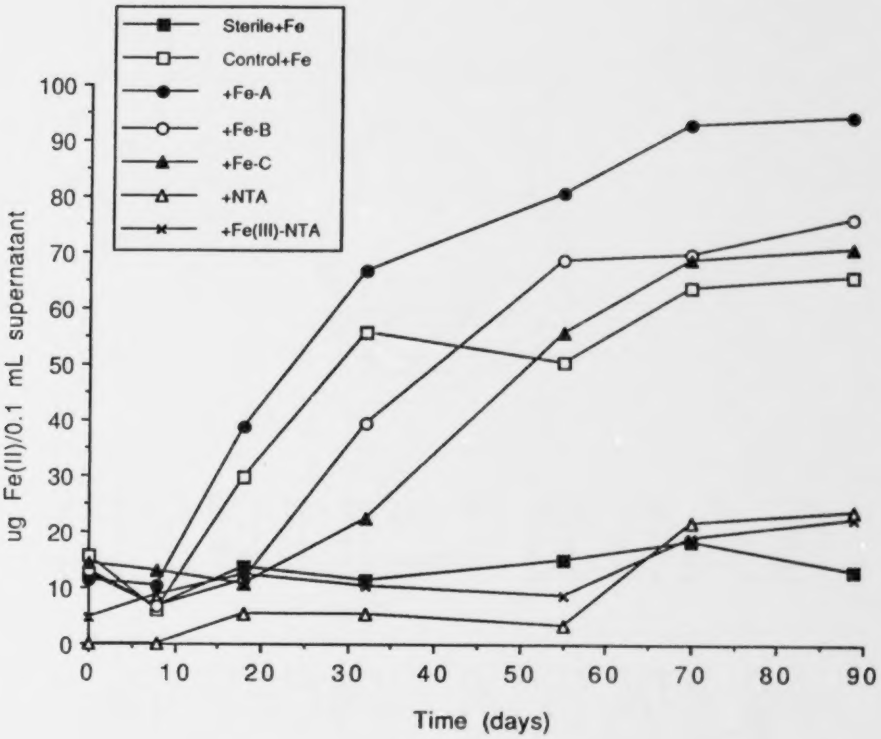

Figure 4.7 Fe(II) production in Borden sand incubated with medium, water-soluble creosote compounds, and $Fe(OH)_3$, NTA, or Fe(III)-NTA. "+Fe" indicates that $Fe(OH)_3$ was added, whereas "+NTA" and "+Fe(III)-NTA" contain only naturally-present Fe(III) to which these substances were added.

the various sediment cultures are presented in Fig. 4.7. The controls and cultures to which no $Fe(OH)_3$ was added showed little accumulation of Fe(II) (data not shown), even in the presence of NTA or Fe(III)-NTA after 89 days of incubation (Fig. 4.7). The three replicate cultures which contained $Fe(OH)_3$ and aqueous OCT compounds accumulated up to 94 µg (+Fe-A), 76 µg (+Fe-B), and 70.5 µg (+Fe-C) Fe(II)/0.1 mL culture during 89 days of incubation. The substrate-free control accumulated 65.8 µg Fe(II)/0.1 mL culture during this time. The average Fe(II) accumulation in the culture bottles containing the OCT compounds (80.2 µg/0.1 mL culture, ± 12.5) was not significantly higher (95% confidence limits) than the Fe(II) accumulation in the control which only contained H_2O.

In an effort to determine the loss of OCT compounds from Borden sand and transfer cultures, controls containing 10 mL medium and $Fe(OH)_3$, 1.0 mL H_2O, and 1.0 mL of the aqueous OCT fraction were acidified and extracted with dichloromethane. Organic extracts were concentrated and analyzed by gas chromatography, but virtually no peaks were detected. Thus, Borden cultures were not analyzed for the loss of aqueous OCT compounds.

4.4 DISCUSSION

Numerous studies in the last decade have shown that microbial populations are present in sedimentary environments which are capable of degrading organic matter under iron-reducing conditions and obtaining energy for growth (Lovley, 1991). Although many of these studies have used sediments from pristine environments, there have been only few reports of microbial iron reduction acitivity in petroleum- and landfill leachate-contaminated sediments. A zone high in iron concentration has been identified at a creosote-contaminated aquifer in Pensacola, Florida (Baedecker and Lindsay, 1986). Anaerobic incubations of sediments from this zone showed an accumulation of Fe(II) over time which suggested that the Fe(III) present could serve as an electron acceptor for microbial degradation of creosote compounds (Lovley and Phillips, 1987b). The preliminary laboratory experiments done in this study showed that both clean and creosote-contaminated sediments from a shallow sandy aquifer near Borden, Ontario contain microbial populations which have iron-reducing capabilities.

The initial experiments with Borden sand sampled from an aerobic portion of the aquifer (aerobic core #1) showed an increase in Fe(II) production over time in the presence of added electron donors and poorly crystalline $Fe(OH)_3$. This iron source was chosen for the Borden sand experiments because it was found in experiments done by Lovley and Phillips (1986a) that iron-reducing populations from sediments could readily use Fe(III) in this form but not when highly crystalline Fe(III) forms were added. Furthermore, $Fe(OH)_3$ is in a form which closely resembles amorphous Fe(III) naturally present in sediments (Lovley *et al.*, 1991). An increase in Fe(II) was also observed in Borden sediments to which O_2-free H_2O and $Fe(OH)_3$ were added, which suggested that there was organic matter naturally present in the sediments which served as an electron donor for iron reduction. Fe(II) production was not observed when sterile sediments were used, indicating that Fe(III) reduction in these sediments was a microbially-mediated process. Although substrate concentrations were not measured, an increase in Fe(II) observed with each subsequent transfer of the phenol-enriched culture suggested that the microbial population was growing by coupling the oxidation of phenol to Fe(III) reduction, which has been shown to be an energetically-favorable reaction (Lovley and Lonergan, 1990).

Table 4.1 shows the theoretical, stoichiometric mineralization reactions of the all substrates used in the iron reduction studies with Borden aquifer material. The thermodynamic calculations ($\Delta G^{\circ\prime}$) indicate that these reactions are energetically-favorable, assuming a suitable iron-reducing microbial population and the correct proportions of eletron donor and electron acceptor. The stoichiometries and free energies calculated for acetate, benzoate, phenol, *p*-cresol, and toluene in Table 4.1 agree closely with those that have been previously calculated for metabolism of these compounds under iron-reducing conditions (Lovley and Phillips, 1988; Lovley and Lonergan, 1990). The actual

Table 4.1 Stoichiometry and standard free energies[a] of mineralization reactions of substrates used in the Borden sand iron reduction studies.

COMPOUND	STOICHIOMETRIC REACTION	$\Delta G^{\circ\prime}$ (kJ/mol)
Acetate	$C_2H_3O_2^- + 4H_2O + 8Fe^{3+} \rightarrow 2HCO_3^- + 9H^+ + 8Fe^{2+}$	-814
Propionate	$C_3H_5O_2^- + 7H_2O + 14Fe^{3+} \rightarrow 3HCO_3^- + 16H^+ + 14Fe^{2+}$	-1427
Butyrate	$C_4H_7O_2^- + 10H_2O + 20Fe^{3+} \rightarrow 4HCO_3^- + 23H^+ + 20Fe^{2+}$	-2039
Lactate	$C_3H_5O_3^- + 6H_2O + 12Fe^{3+} \rightarrow 3HCO_3^- + 14H^+ + 12Fe^{2+}$	-1276
Glucose	$C_6H_{12}O_6 + 12H_2O + 24Fe^{3+} \rightarrow 6HCO_3^- + 30H^+ + 24Fe^{2+}$	-1914
Benzoate	$C_7H_5O_2^- + 19H_2O + 30Fe^{3+} \rightarrow 7HCO_3^- + 36H^+ + 30Fe^{2+}$	-3036
p-Hydroxy-benzoate	$C_7H_5O_3^- + 18H_2O + 28Fe^{3+} \rightarrow 7HCO_3^- + 34H^+ + 28Fe^{2+}$	-2869
Phenol	$C_6H_6O + 17H_2O + 28Fe^{3+} \rightarrow 6HCO_3^- + 34\ H^+ + 28Fe^{2+}$	-2890
p-Cresol	$C_7H_8O + 20H_2O + 34Fe^{3+} \rightarrow 7HCO_3^- + 41H^+ + 34Fe^{2+}$	-3514
Benzene	$C_6H_6 + 18H_2O + 30Fe^{3+} \rightarrow 6HCO_3^- + 43H^+ + 30Fe^{2+}$	-3058
Toluene	$C_7H_8 + 21H_2O + 36Fe^{3+} \rightarrow 7HCO_3^- + 43H^+ + 36Fe^{2+}$	-3652

[a] Free energy calculated using standard free energies of reactants and products from Thauer *et al.* (1977) and Brock and Madigan (1991).

metabolism of the compounds listed in Table 4.1 would be more complex than indicated by the stoichiometric reactions because some carbon would be incorporated into cellular material for growth and Fe(II) and Fe(III) would likely exist in different forms (Lovley and Lonergan, 1990).

Sediments sampled from a different aerobic zone (aerobic core #2) in the Borden aquifer had iron-reducing activity when simple substrates were added along with amorphous $Fe(OH)_3$, but not when aromatic substrates were added. The substrate-free control from these experiments did not show increased Fe(II) production (Fig. 4.1), so the increase in Fe(II) in the lactate-, glucose-, and butyrate-enriched sediments was likely due to dissimilatory iron reduction. Table 4.2 shows the percentage of microbial iron reduction that actually occurred in these cultures, which was calculated from the stoichiometric reactions in Table 4.1 and the amounts of electron donor and electron acceptor added to the culture bottles. The predicted amount of Fe(II) that would be produced (Table 4.2) was calculated by assuming that the cultures were capable of mineralizing the substrate in question, and calculations were made only for those cultures showing Fe(II) production above that of the controls. For the cultures depicted in Fig. 4.1, the electron donor was added in excess of the electron acceptor, so a complete reduction of the added Fe(III) was expected. According to Table 4.2, greater than 100% of the predicted amount of Fe(II) was actually measured from the lactate-enriched culture, whereas only 28% and 34% of the predicted amounts of Fe(II) were measured for the glucose- and butyrate-enriched cultures, respectively.

The lactate-containing culture from aerobic core #2 was the only one which was transferrable from this particular sediment, and it was found that microorganisms derived from this sediment preferred to use insoluble $Fe(OH)_3$ as a Fe(III) source over soluble Fe(III) citrate. A separate experiment in which the sediments were incubated initially in the presence of Fe(III) citrate medium did not show an increase in Fe(II) production for any of the substrates tested (data not shown). Although acetate has commonly been used to enrich

Table 4.2 The percentage of Fe(III) reduced by Borden sand cultures based on predicted and actual amount of Fe(II) produced.

Substrate	Predicted amount Fe(II) produced[a] (μg/0.1 mL culture)	Actual amount Fe(II) produced (μg/0.1 mL culture)	% Fe(III) reduction
Aerobic core #2 (Fig. 4.1)			
Lactate	625	740	> 100
Glucose	625	177	28
Butyrate	625	213	34
Anaerobic core (Fig. 4.2)			
Acetate	407	208	51
Propionate	407	250	61
Butyrate	407	224	55
Glucose	407	212	52
Lactate	407	346	85
Benzoate	407	288	71
p-Hydroxybenzoate	407	91	22
p-Cresol	407	26	6
Toluene	407	27	7
Anaerobic core (Fig. 4.3a)			
Acetate	446	225	59
Glucose	446	429	96
Lactate	446	241	54
Benzoate*	281	301	> 100

[a] Based on stoichiometries from Table 4.1 and amounts of electron donors and $Fe(OH)_3$ added to the culture bottles. In all cases except *, the electron donor was added in excess, thus Fe(III) was the limiting factor.

for iron-reducing bacteria in sediments (Lovley and Phillips, 1988; Lovley *et al.*, 1990; Lovley *et al.*, 1991), acetate did not serve as an electron donor for Fe(III) reduction in these sediments. The lack of iron-reducing activity observed when these sediments were incubated with aromatic compounds may have been due to a number of reasons. It is possible that the sediments used for this experiment did not contain a population of iron-reducing bacteria, that the concentration of substrates added was toxic to the microorganisms, or that the iron-reducing population present did not have the genetic capabilities to oxidize aromatic compounds. This latter reason is the most probable explanation because these sediments were sampled from a portion of the Borden aquifer which had received no prior exposure to aromatic pollutants.

Borden sediments which were incubated at 35°C did not show signs of iron-reducing activity except for the sediments to which glucose was added in the presence of $Fe(OH)_3$. Considering that these sediments were removed from the Borden aquifer which has an average temperture of 12°C, this is not too surprising. Incubating Borden sediments at room temperature (about 22-24°C) for all other experiments, however, did result in iron-reducing activity. In sediments to which *G. metallireducens* was added, there was an increase in Fe(II) production for all of the substrates tested, but to the same level as the substrate-free control (except with toluene). This, and the fact that no decrease in substrate concentrations occurred, indicated that *G. metallireducens* was not oxidizing the added substrates under iron-reducing conditions in the presence of the Borden sediments. Separate experiments with *G. metallireducens* had verified that this bacterium was capable of degrading many organic substrates in the presence of $Fe(OH)_3$, as has been reported (Lovley *et al.*, 1993). Thus, the ability of this iron-reducing organism to oxidize organic compounds under iron-reducing conditions was somehow inhibited by incubation with the Borden sediments. Because these sediments were derived from an aerobic portion of the aquifer, they may have contained concentrations of oxygen which were lethal to this strict anaerobe, though dissolved oxygen concentrations were not measured for any of the

Borden sand experiments. These observations are in contrast to previous work in which *G. metallireducens* showed iron-reducing activity when added to sediments with benzoate as an electron donor (Lovley *et al.*, 1989b).

An interesting observation from these initial experiments was that a color change of the $Fe(OH)_3$ layers in the microcosms was associated with an increase in Fe(II) production. In all of the sterile controls, the $Fe(OH)_3$ layer remained rusty-colored, whereas the $Fe(OH)_3$ layers containing live sediments changed to brown or gray. Color changes have been observed in experiments with other iron-reducing bacteria. For example, the growth of *G. metallireducens* or *G. sulfurreducens* on acetate using $Fe(OH)_3$ as an electron acceptor resulted in the formation of an ultra-fine-grained black magnetic material which was identified as magnetite, a mixed Fe(III)-Fe(II) mineral (Fe_3O_4) (Lovley and Phillips, 1988; Caccavo *et al.*, 1992). When *G. metallireducens* was incubated with acetate and $Fe(OH)_3$ in our laboratory, however, a black, magnetic precipitate did not form, although the $Fe(OH)_3$ layer did change from rusty-colored to brown. When *G. metallireducens* was incubated with Borden sediments, a brown layer formed as well with most of the substrates. The one exception was the glucose-containing cultures, in which a grayish-white precipitate formed when $Fe(OH)_3$ was added to the sediments in the presence or absence of *G. metallireducens*. When these sediment cultures were tranferred to Fe(III) citrate medium amended with glucose, a white precipitate formed again, and the orange color of this medium turned clear. The formation of a white precipitate under these conditions occurred with all subsequent transfers of these cultures. Although not analyzed for, this white precipitate was presumed to be vivianite, a Fe(II) mineral [$Fe_3(PO_4)_2{\cdot}8H_2O$]. Vivianite was produced by *G. metallireducens* incubated in Fe(III) medium with acetate as an electron donor (Lovley and Phillips, 1988), which was also observed in our laboratory. (Unusual observations with the well-characterized *G. metallireducens* obtained from ATCC led to the question of the purity of this organism. This culture was found to be impure after streaking on plates and doing wet mounts, but it is not clear whether it was received impure from ATCC or whether this culture was contaminated in our laboratory).

Similar color changes were observed when sediments sampled from an anaerobic portion of the Borden aquifer were incubated with different electron donors in the presence of $Fe(OH)_3$. Along with these color changes, an increase in Fe(II) was evident when non-aromatic substrates were present compared to a substrate-free control (Fig. 4.2a), which suggested these sediments contained iron-reducing populations which could couple the oxidation of these substrates to the reduction of Fe(III). This suggestion had been made in a previous study which demonstrated an accumulation of Fe(II) when anaerobic sediments were incubated with $Fe(OH)_3$ in the presence of similar compounds (Lovley and Phillips, 1986). The percentage of microbial Fe(III) reduction that actually occurred in the anaerobic Borden sand cultures in Fig. 4.2a are listed in Table 4.2, ranging from 51% for acetate to 85% for lactate. Even though these Borden sediments had been sampled from a creosote-contaminated zone (which would be enriched in aromatic compounds), significant iron reduction was observed only when benzoate was added as the electron donor (Fig. 4.2b). Table 4.2 shows that about 71% of the added Fe(III) to Borden sand containing benzoate was reduced, whereas less than 25% Fe(III) was reduced when *p*-hydroxybenzoate, *p*-cresol, or toluene were added as electron donors. Although benzoate was postulated to be an intermediate in the metabolism of phenol, *p*-cresol, and *p*-hydroxybenzoate under iron-reducing conditions (Lovley and Lonergan, 1990), incubation of a benzoate-enriched culture from Borden anaerobic sediments with these aromatic compounds under iron-reducing conditions did not result in their oxidation or in Fe(III) reduction.

When another experiment with the anaerobic Borden sand was set up with acetate, lactate, glucose, or benzoate as electron donors and $Fe(OH)_3$ as the electron acceptor, different amounts of Fe(III) reduction occurred (Fig. 4.3a) than had been observed previously (Fig. 4.2). For example, Table 4.2 shows that in the culture containing glucose from Fig. 4.2, 52% of the added Fe(III) was reduced, whereas in the culture containing glucose from Fig. 4.3a, 96% of the added Fe(III) was reduced. Differences were also observed for lactate and benzoate for the two experiments. Because of these differences, it is difficult to come to any conclusion as to which substrates are best degradable under iron-reducing conditions by the microbial populations in the Borden aquifer. It is possible that different populations existed within the same core sample, thus giving rise to different amounts of iron reduction in different experiments to which the same electron acceptor was added.

In all of the Borden sand experiments, only small increases in Fe(II) were observed when no $Fe(OH)_3$ was added prior to incubation with electron donors, in comparison to $Fe(OH)_3$-amended cultures. Using hydroxylamine-HCl as a reducing agent, it was found that Fe(III) was present in the Borden core samples which was available for use by microorganisms. Hydroxylamine-HCl has been shown to be useful in determining whether naturally-present Fe(III) oxides can be readily used as terminal electron acceptors for iron-reducing populations in sediments (Lovely and Phillips, 1987b). Thus, it was puzzling that such a small amount of iron reduction was observed when only the naturally-present Fe(III) in the Borden aquifer material was available. In a recent study which examined the potential for microbial iron reduction in landfill leachate-contaminated sediments, Fe(III) naturally present in the sediments was reduced (Albrechtsen and Christensen, 1994). Using unamended sediments and groundwater, these researchers observed an increase of only 2 to 7 µg/g dry weight sediment in addition to the control which was considered significant. Thus the increases in Fe(II) in the unamended anaerobic Borden sediments ranging from 4 to 18 µg Fe(II)/0.1 mL culture supernatant could be deemed significant as well. However, the addition of $Fe(OH)_3$ to the sediments resulted in at least 10-fold higher amounts of Fe(II) in the sediment cultures.

It is possible that measurements of dissolved Fe(II) in the Borden sediments cultures could have been underestimated because Fe(II) produced in sediments as a result of microbial iron reduction can bind to sediment, undergo ion exchange, become precipitated as carbonates, or become part of an iron mineral. According to Lovley (1991), most of the Fe(II) produced as a result of microbial iron reduction in sediments is not recovered as dissolved Fe(II) but remains in solid mineral forms, like siderite, vivianite, and mixed Fe(II)-Fe(III) minerals like magnetite. In studies with iron reduction in crude oil-contaminated sediments, Baedecker *et al.* (1993) found that much of the ferrous iron produced was precipitated as carbonates. The formation of brown, gray, or white precipitates in many of the Borden sediment cultures suggests that either Fe(II) or mixed Fe(III)-Fe(II) minerals are being produced as a result of microbial iron reduction. These colored precipitates were not observed in sterile controls.

Preliminary experiments with the anaerobic Borden sediments also showed iron reduction activity in the presence of water-soluble creosote compounds such as phenols, nitrogen-heterocycles, and low-molecular-weight polycyclic aromatic hydrocarbons. Increases in Fe(II) were observed when Borden sediments and subsequent transfer cultures were incubated with $Fe(OH)_3$ and water-soluble OCT. Benzoate-enriched transfer cultures did not show iron reduction activity in the presence of these compounds, while acetate-, lactate-, glucose-, and butyrate-enriched cultures did (Figs. 4.5 and 4.6). Borden sediments incubated in the presence of water-soluble OCT components and $Fe(OH)_3$ showed small increases in Fe(II) production over that of sediments containing no substrate (Fig. 4.7) after 89 days of incubation. An increase in Fe(II) production seen with the control to which the water-soluble OCT fraction was not added may be explained by the fact that the anaerobic core material was sampled from within the contaminated plume, and thus likely contains water-soluble creosote compounds which could serve as electron donors under iron-reducing conditions. Prolonged incubation may enhance the difference between the Fe(II) produced in OCT-containing sediments and substrate-free control sediments.

Although it was suggested earlier that microbial populations in the Borden sediments were coupling the oxidation of organic compounds with the reduction of Fe(III), the results presented here do not indicate that Fe(III) was the sole terminal electron acceptor or that this was an energy-yielding process. Growth of cultures was not measured, and in many cases, substrate loss was not monitored. More in-depth analyses are required to more clearly understand the iron reduction processes occurring in the Borden sediments. These include monitoring growth, substrate loss, loss of Fe(III), and identification of the Fe(II) and/or Fe(II)-Fe(III) minerals being formed. Experiments with ^{14}C-labelled substrates would determine whether the substrates were being mineralized under iron-reducing conditions, and the isolation of pure iron-reducers from these sediments would enable mass balances in the metabolism of carbon sources under iron-reducing conditions.

Nevertheless, the accumulation of Fe(II) in cultures amended with $Fe(OH)_3$ and different substrates including water-soluble creosote components above that of substrate-free controls and sterile controls indicated that iron-reducing populations are present in the Borden aquifer. Although the results presented here do not reflect *in situ* processes, they do suggest that microbial iron reduction in the Borden aquifer could be an important process in the degradation of creosote compounds but more laboratory experiments and subsequent field experiments are necessary to confirm this.

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5. AEROBIC BIODEGRADATION OF CARBAZOLE, A NITROGEN-HETEROCYCLE FOUND IN COAL TAR CREOSOTE*

5.1 INTRODUCTION

Nitrogen-, sulfur-, and oxygen-heterocycles have been found in soils and groundwaters at sites contaminated with petroleum and wood-preserving wastes (Pereira *et al.*, 1987; Mueller *et al.*, 1989; Godsey *et al.*, 1989; Bollag and Kaiser, 1991), and are of concern because of their toxicities and recalcitrance. Although many of these heterocycles have been found to be biodegraded or biotransformed in laboratory cultures (Berry *et al.*, 1987; Foght and Westlake, 1988; Arvin *et al.*, 1989; Bollag and Kaiser, 1991), little is known about their fate in the environment (Pereira *et al.*, 1987; Mueller *et al.*, 1989). Some laboratory and field studies have examined the fate of N-heterocycles in a creosote-contaminated aquifer (Bennett *et al.*, 1986; Pereira *et al.*, 1987; Pereira *et al.*, 1988a; Pereira *et al.*, 1988b). Of the N-heterocycles present in creosote and petroleum wastes, the biodegradation of quinoline and its methyl derivatives has been the most frequently studied (Shukla, 1986; Pereira *et al.*, 1988a; Pereira *et al.*, 1988b; Dembek *et al.*, 1989; Aislabie *et al.*, 1990; Miethling *et al.*, 1993).

Carbazole has received relatively less attention. Like quinoline, carbazole has been found to be a predominant N-heterocycle in coal tar creosote (Mueller *et al.*, 1989) and found to be present in groundwater and soil contaminated with creosote (Pereira *et al.*, 1987; Mueller *et al.*, 1991a, Mueller *et al.*, 1991b; Fowler *et al.*, 1994). Although considered a recalcitrant compound in groundwater systems under anaerobic conditions (Pereira *et al.*, 1987; Liu *et al.*, 1994), carbazole is biodegradable by microorganisms under aerobic conditions. Bohonos *et al.* (1977) observed carbazole biodegradation in various contaminated water samples when carbazole was added at concentrations ranging from 10 to 100 μg/mL, although no metabolites were detected. Finnerty *et al.* (1983) used carbazole as a model compound to study the microbial denitrogenation of petroleum materials. Four bacteria were isolated from soil that could use carbazole as a sole source of carbon and energy, one of which could also use this compound as a sole nitrogen source. Several water-soluble products of carbazole dissimilation were detected by thin-layer chromatography, although these were not identified. Fedorak and Westlake (1984) reported that carbazole enrichment cultures were capable of degrading a wide range of alkyl carbazoles present in Norman Wells crude oil. Using ^{14}C-labelled carbazole, Grosser *et al.* (1991) reported the mineralization of carbazole in soils which had been inoculated with a previously isolated carbazole-degrading *Xanthomonas* species, whereby 45% of the label was recovered as $^{14}CO_2$. Recently, Ouchiyama *et al.* (1993) proposed a pathway for the metabolism of carbazole by two *Pseudomonas* isolates, although only anthranilic acid and catechol were positively identified as metabolites. Resnick and Gibson (1993) also reported anthranilic acid as a metabolite of carbazole degradation as well as 1-hydroxycarbazole formation by a mutant *Pseudomonas* strain.

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As part of a 5-year project examining the fate of creosote compounds in groundwater at a well-characterized shallow, sandy aquifer at CFB Borden, Ontario, the aerobic biodegradation of carbazole was studied, mainly because little was known about the biodegradation of this N-heterocycle. Carbazole was added to the commercial coal tar creosote used for the field experiment (3 g/kg creosote) and monitoring of its dissolution from the creosote source and migration through the groundwater is ongoing (Fowler *et al.*, 1994; King *et al.*, 1994).

At the time this research project began (1991), no intermediates of carbazole metabolism had been reported, and the goal of this investigation was to enrich for bacteria which could degrade carbazole and to identify some of the metabolites. Mixed carbazole-degrading cultures were established, and a carbazole-degrading bacterium was isolated in pure culture. The ability of the isolate to grow on or transform a variety of aromatic compounds, carbazole analogs, and possible intermediates was tested to help elucidate the manner in which carbazole was metabolized. This chapter describes some of the metabolic capabilities of a bacterium that uses carbazole as its sole source of carbon, nitrogen, and energy. In addition, several metabolites of carbazole degradation were identified.

5.2 MATERIALS AND METHODS

See Appendix A for media compositions and the sources of all chemicals used in these experiments.

5.2.1 Growth media and conditions

Experiments examining the biodegradation of carbazole were generally done in 500-mL Erlenmeyer flasks containing 200 mL of a nitrogen-free mineral medium, denoted B-N. For some experiments, B+N medium was used, which contained the inorganic nitrogen sources, KNO_3 and NH_4Cl. Carbazole and other substrates were usually added directly to culture media in their crystalline forms. All cultures were incubated at 28°C in the dark on a rotary shaker at 200 rpm, unless otherwise noted. Carbazole-degrading cultures were routinely maintained in 200 mL of B-N medium containing 50 mg of carbazole, and a 10% inoculum (by volume) was used for transfers. Glycerol stocks were also made for each carbazole-degrading population and stored at -70°C.

Plate count agar (PCA) was used to isolate carbazole-degrading bacteria and to enumerate bacteria by the plate count method. Agar plates were incubated at 28°C for at least 3 days before counting. Minimal medium plates containing carbazole as well as BYP plates containing the antibiotic kanamycin (Km) were also used for some experiments with carbazole-degrading bacteria .

5.2.2 Enrichments for carbazole-degrading populations

A carbazole-degrading population (CM, for Carbazole Maintenance) which had long been maintained in our laboratory was initially used to study carbazole degradation. As well, a carbazole-degrading population was enriched from creosote-contaminated soil from a site in Edmonton, Alberta. Soil (10 g) was added to Stanier's mineral medium and incubated with 20 mg carbazole. This culture was transferred every 4 days for 16 days into fresh medium and substrate before being transferred into B-N medium with 0.1 g carbazole. This mixed carbazole-degrading culture was designated DC. Both the CM and DC cultures were maintained with weekly transfers with carbazole as the sole carbon and nitrogen source, and used as sources to look for pure carbazole-degrading organisms.

Two carbazole-degrading populations were also obtained from J. Aislabie (Landcare Research N.Z. Ltd., Waikato, Hamilton, New Zealand), which were designated CB1, a *Comamonas* sp., and CB2, a *Pseudomonas* sp. (Aislabie, 1994a). These bacteria were not pure upon arrival at our laboratory, and attempts were made to purify them. A pure culture of CB1 was obtained, although this bacterium did not appear to degrade carbazole well, and it was not possible to purify CB2 and retain carbazole-degrading ability. Thus, CB1 and CB2 were maintained as mixed cultures and were designated CB1 Mix and CB2 Mix. The pure CB1 culture was also maintained and was designated CB1 Pure. These cultures absolutely required the addition of yeast extract to B-N medium for growth. The designations of cultures used for carbazole-degrading experiments are summarized in Table 5.1.

5.2.3 Isolation of a carbazole-degrading bacterium

The mixed carbazole-degrading cultures CM and DC were streaked on PCA and five morphologically different colonies from each mixed culture were selected and tested for their abilities to degrade carbazole in B-N medium. The visual loss of carbazole

Table 5.1 Summary of pure and mixed cultures used in carbazole biodegradation experiments.

Culture Name	Pure or Mixed	Identification	Source
CM	mixed	unidentified bacterial population	Edmonton
DC	mixed	unidentified bacterial population	Edmonton
D5	mixed	two unidentified bacteria	Edmonton
LD2	pure	*Pseudomonas* sp.	Edmonton
CB1 Pure	pure	*Comamonas* sp.	New Zealand
CB1 Mix	mixed	*Comamonas* sp. & unidentified bacterium	New Zealand
CB2 Mix	mixed	*Pseudomonas* sp. & unidentified bacterium	New Zealand
PMB 11	pure	LD2 mutant[a]	Texas
PMB 12	pure	LD2 mutant	Texas
PMB 13	pure	LD2 mutant	Texas
PMB 17	pure	LD2 mutant	Texas
PMB 18	pure	LD2 mutant	Texas
PMB 19	pure	LD2 mutant	Texas
PMB 20	pure	LD2 mutant	Texas
PMB 21	pure	LD2 mutant	Texas
PMB 22	pure	LD2 mutant	Texas

[a] transposon (Tn5) mutant

crystals as well as streaking for growth after repeated transfers were used as criteria to determine whether the selected colony type was able to utilize carbazole as the sole carbon and nitrogen source. Of the 10 originally chosen, only 2 colony types were found which grew on carbazole in B-N medium. These were named LD2 and D5, and were isolated from the mixed culture, DC. Although D5 was originally pure, a persisent contaminant came to be associated with this organism even after repeated attempts at repurification. Thus, D5 was designated a mixed culture (containing two different morphologically different bacteria on PCA) and maintained as such. Isolate LD2, however, remained pure, and was subsequently characterized by various taxonomic tests. This carbazole-degrading bacterium was used for most of the carbazole degradation studies described here.

The ability of isolate LD2 to fix N_2 was evaluated using the acetylene reduction assay (Turner and Gibson, 1980). In this assay, acetylene was added to serum bottles containing B-N medium, carbazole, and isolate LD2, then sealed with rubber stoppers. The production of ethylene and the loss of acetylene were monitored over time by removing samples and analyzing by GC. *Azotobacter vinelandii*, a known N_2-fixing bacterium (obtained from Dr. W.J. Page, Department of Biological Sciences, University of Alberta), was used as a positive control and yielded ethylene under the experimental conditions.

5.2.4 Transposon mutants of LD2

As part of a collaborative project, transposon (Tn5) mutants of LD2 (designations in Table 5.1) were made by R. Riddle and Dr. M. Benedik in the Department of Biochemical and Biophysical Sciences at the University of Houston, Texas. The mutants were selected for Km resistance (from Tn5) and for the reduced ability to clear carbazole-containing minimal medium plates. The transposon was stable, so it was not necessary to use medium containing Km. Upon arrival in our laboratory, the mutants were streaked on BYP plates containing Km to verify this antibiotic resistance and were also streaked on carbazole-containing minimal medium plates to examine carbazole degrading ability compared to wild type LD2. Washed cells of LD2 mutants were incubated with carbazole for 24 h and culture supernatants were examined for products of carbazole degradation using methods described in Section 5.2.9.

5.2.5 Substrates tested for the growth of isolate LD2

The following compounds were tested as growth substrates for isolate LD2 in the presence and absence of carbazole: 9-methylcarbazole, 2-hydroxycarbazole, 1,2,3,4-tetrahydrocarbazole, 2-aminobiphenyl, 2-nitrobiphenyl, phenothiazine, iminostilbene, fluorene, dibenzofuran, dibenzothiophene, anthranilic acid, and indole-3-acetic acid. Other substrates tested as carbon sources included: glucose, acetate, succinate, pyruvate, catechol, salicylate, gentisate, benzoate, benzene, naphthalene, phenanthrene, biphenyl, 2,2'-dihydroxybiphenyl, 2,3-dihydroxybiphenyl, indole-3-pyruvic acid, indole-3-propanoic acid, indole-3-butanoic acid, indole-3-carboxaldehyde, indole-3-carboxylic acid, *trans*-indole-3-acrylic acid, L-tryptophan, DL-kynurenine, and indole.

In the growth experiments without carbazole in the medium, isolate LD2 was inoculated into B+N and/or B-N medium containing 20 to 50 mg of the desired compound. B+N medium was used if the compound being tested did not contain a nitrogen atom in its molecular structure. If carbazole was present, B-N medium was used. The cultures were incubated from 2 to 4 weeks, then acidifed and extracted with ethyl acetate or dichloromethane (CH_2Cl_2) for gas chromatographic analysis to determine whether the compound had been consumed or transformed. Whenever cultures of isolate LD2 were incubated in the presence of an aromatic compound to test for growth or biotransformation, appropriate sterile controls were incubated and analyzed in the same manner as the test cultures to account for any abiotic transformations. None of the transformation products discussed in these experiments was found in the sterile controls.

5.2.6 Mineralization of radiolabelled carbazole and other substrates

The mineralization of [U-^{14}C]carbazole by isolate LD2, and the mixed cultures CM, DC, and D5 was studied using 500-mL Erlenmeyer flasks equipped with side arms for sampling. Flasks containing 200 mL B-N medium were incubated with the respective carbazole-degrading populations in the presence of 50 mg carbazole and approximately 400, 000 dpm [U-^{14}C]carbazole (^{14}C-carbazole in CH_2Cl_2 was mixed with "cold" carbazole in the side arm flask and the solvent was allowed to evaporate prior to addition of medium and inoculum). At various times, liquid (5 mL) and headspace air (10 mL) from the flasks were sampled and added to pre-evacuated 60-mL serum bottles. The samples were acidified and flushed into scintillation vials containing 10 mL ACS fluor and 1 mL Carbo-Sorb II to recover $^{14}CO_2$ using the trapping apparatus described by Fedorak *et al.* (1982). The amount of radiolabel in the vials was counted by liquid scintillation and the percentage of label recovered as $^{14}CO_2$ was calculated based on the amount of radiolabel originally added. Sterile controls were set up as well.

Mineralization of [U-^{14}C]carbazole, [U-^{14}C]glucose, and [2-^{14}C]acetate by isolate LD2 was studied using the same method. Cultures contained a mixture of both non-radiolabelled and radiolabelled substrates, and radioactivity levels were approximately 400,000 dpm per culture. Mineralization experiments were done with glucose or acetate supplied as the major carbon source and carbazole added as the nitrogen source in the following manner: (a) radiolabelled carbazole plus non-radiolabelled glucose or acetate and (b) non-radiolabelled carbazole plus radiolabelled glucose or acetate. The carbon to nitrogen ratio in these experiments was 200:1 on a molar basis. Flasks were incubated and at various times, the liquid and gas phases were sampled to recover $^{14}CO_2$ that had been produced (Fedorak *et al.*, 1982). These experiments done with B-N and B+N media.

The ability of isolate LD2 to mineralize five hydrocarbons, [U-^{14}C]biphenyl, [1-^{14}C] naphthalene, [9-^{14}C]phenanthrene, [9-^{14}C]anthracene, and *n*-[1-^{14}C]hexadecane was tested using the method of Foght *et al.* (1990). Briefly, 1-mL portions of a suspension of isolate LD2 were added to 158-mL serum bottles containing 10 mL of B+N medium and 10 μL of Norman Wells crude oil, supplemented with approximately 10,000 dpm of a radiolabelled substrate. Six serum bottles were set up for each substrate: two were analyzed after 14 days incubation, two were analyzed after 28 days incubation, and two served as sterile controls. Cultures of isolate LD2 with oil and ^{14}C-carbazole were monitored in the same manner. After appropriate incubation times, cultures were acidified and flushed to trap $^{14}CO_2$, as above.

5.2.7 Extraction of metabolites from carbazole-degrading cultures

Metabolites from the degradation of carbazole or other substrates were extracted from the culture medium with ethyl acetate either at neutral pH or after acidification to pH < 2 with 4 N H_2SO_4. Organic extracts were dried over sodium sulfate, concentrated to small volumes by rotary evaporation, and frequently derivatized using one of the methods described below prior to analysis by GC. For samples used to quantify the loss of carbazole by GC, *n*-hexadecane was added as an internal standard before extraction with ethyl acetate and derivatization was not performed. Samples analyzed by high performance HPLC were not derivatized, and were dissolved in methanol (HPLC grade) prior to analysis.

5.2.8 Derivatization methods

In order to detect polar metabolites by GC, four methods of derivatization were used. Some samples were dissolved in *N, N*-dimethylformamide (DMF) for derivatization reaction with *N, O*-bis-(trimethylsilyl)acetamide (BSA) (Pierce, 1989). This involved mixing equal volumes of sample solution and BSA and heating at 70°C. Samples were cooled before GC analysis. In some cases, an ethereal solution of diazomethane was used to derivatize samples. Diazomethane was prepared from *N*-methyl-*N*-nitroso-*N*-nitroguanidine using a millimole size generator according to manufacturer's instructions

(Wheaton, Millville, NJ) and was added to concentrated organic extracts immediately after preparation. Reacted samples were allowed to sit overnight before GC analysis.

A solution of acetic anhydride and pyridine (3:2 vol/vol) was used to acetylate dried organic extracts from cultures (Coutts *et al.*, 1991). A BF_3-methanol complex was also used to derivatize organic culture extracts. In this method, 1.0 mL BF_3-methanol was added to dried organic extracts and the mixture was heated at 60°C for 15 min. After cooling, the reaction mixture was diluted to 10 mL with CH_2Cl_2, extracted with 10 mL 10% NaCl in water, and the CH_2Cl_2 layer was dried over Na_2SO_4. The aqueous layer was re-extracted with CH_2Cl_2, and the two CH_2Cl_2 layers were combined, dried, and redissolved in a small volume of ethyl acetate for GC analysis.

5.2.9 Washed cell suspension experiments

Strain LD2 was incubated for 40 h with carbazole, harvested by centrifugation at 10,000 rpm for 10 min, and washed with B-N medium. Supernatants were saved. A cell suspension (OD_{600} of 1.42) was added to 50 mL of B-N medium containing 20 mg carbazole and incubated. A sterile control and heat-killed cell control were also prepared. After 3 or 7 h, these three preparations were sonicated using a Sonifier Cell Disruptor 350 with a 0.75 inch diameter probe (Branson Sonic Power Co., Danbury, CT). The resulting suspension was extracted with ethyl acetate under neutral and acidic conditions, and the organic extracts were concentrated to small volumes by rotary evaporation. The residue was dissolved in methanol for HPLC analysis or in DMF for derivatization with BSA for GC analysis. The saved supernatants were also extracted under neutral and acidic conditions and prepared in the same way for HPLC and GC analyses.

For induction experiments, isolate LD2 was incubated with glucose, anthranilic acid, or carbazole to early stationary phase. Cells were centrifuged and washed with B-N medium before being added to flasks containing 50 mL B-N medium and 30 mg anthranilic acid in the presence (100 or 200 μg/mL) or absence of chloramphenicol (CAP). At various times during incubation, 1.0 mL samples were removed, centrifuged, and analyzed directly by HPLC for the loss of anthranilic acid. Multiple flasks containing 20 mg carbazole in the presence or absence of 100 µg/mL CAP were also incubated with glucose- or anthranilic acid-grown LD2 cells. These were extracted at various times under acidic conditions with ethyl acetate and the concentrated ethyl acetate layers were analyzed by GC to observe the loss of carbazole. *n*-Hexadecane was added as an internal standard prior to extraction in order to quantify carbazole degradation.

LD2 mutants and wild type LD2 were grown for 24 h in tryptic soy broth (TSB). Cells were centrifuged and washed with B-N medium before being incubated in B-N medium containing carbazole. After 24 h, the cultures were centrifuged and the supernatants were acidified and extracted with ethyl acetate. The organic extracts were treated with diazomethane and analyzed by GC to compare the carbazole metabolites produced by wild-type LD2 and the LD2 mutants.

5.2.10 Enzyme assays

Both catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activities in isolate LD2 were measured spectrophotometrically according to the methods of Gibson (1970). LD2 cells incubated with carbazole were harvested in late log phase by centrifugation and washed twice with potassium phosphate buffer (20 mM, pH 6.8). Cells in 5 mL of buffer were broken by sonication, and the resulting suspension was centrifuged. The pellet was discarded and the supernatant was kept on ice for protein measurements and enzyme assays. Total protein was measured using the standard procedure described by Bio-Rad Laboratories (Richmond, CA) with bovine serum albumin as the standard.

For the catechol 1,2-dioxygenase assay, the reaction mixture contained 1.0 mL potassium phosphate buffer (0.1 M, pH 7), 200 µL 0.01 M EDTA, 50 µL H_2O, and 100 µL supernatant (containing 89 µg protein). The reaction was started by the addition of 150 μL catechol (prepared as a 0.01 M solution in 95% ethanol) and the change in absorbance at 260 nm was measured over time due to formation of *cis, cis*-muconic acid.

Catechol 2,3-dioxygenase was assayed by measuring the formation of 2-hydroxymuconic semialdehyde. The reaction mixture contained 625 μL potassium phosphate buffer (0.5 M, pH 7), 625 μL buffer with 10% acetone (by volume), and 100 μL supernatant. Catechol (150 μL) was added to initiate the reaction and the change in absorbance at 375 nm was measured over time. Specific activities are expressed as μmol substrate transformed/min/mg protein.

5.2.11 Purification of carbazole metabolites by TLC

Two carbazole metabolites (compounds **VI** and **X**) were purified as methylated derivatives by preparative thin-layer chromatography (TLC) using silica gel plates (1000 μm thickness) with a fluorescence indicator (J.T. Baker, Phillipsburg, NJ). The plates were developed with benzene:acetone:acetic acid (90:15:5). Separated bands were eluted from scraped silica gel with CH_2Cl_2 and concentrated to small volumes to verify purity by GC and TLC.

TLC was also used to visualize catechol formation from extracts of isolate LD2 grown on carbazole or anthranilic acid. Silica gel plates (250 μm thickness) with a fluorescence indicator were used, and were developed with benzene:methanol:acetic acid (90:8:16). Under these conditions, catechol had an Rf value of 0.66.

5.2.12 Chemical syntheses

Using indole-3-acetic acid as the starting material, 2-(2-oxo-indole-3-yl)acetic acid (referred to as oxindole-3-acetic acid) was synthesized by the method of Takase *et al.* (1986). Briefly, indole-3-acetic acid (3 g) was stirred with 12.2 mL dimethylsulfoxide and 28.8 mL concentrated HCl was slowly added. After 15 min, the reaction was diluted with H_2O and extracted with ethyl acetate. Ethyl acetate was removed by rotary evaporation and the residue was recrystallized with acetone-benzene (3:1 vol/vol). The white crystals that formed had a melting point of 147-148°C (literature 146°C). Oxindole-3-propanoic (m.p. 172-174°C) and oxindole-3-butanoic acid (m.p. 175-178°C) were synthesized in the same manner from indole-3-propanoic and indole-3-butanoic acids, respectively.

5-(2-Aminophenyl)-5-oxo-pentanoic acid was synthesized in impure form via the periodate oxidation of indole-3-butanoic acid in a two step reaction (Rivett and Wilshire, 1971). The first step involved adding 14 mmol sodium periodate in 20 mL H_2O to a rapidly stirring solution of 5 mmol indole-3-butanoic acid in 20 mL methanol. After stirring for 20 h, the reaction mixture was filtered and the filtrate was concentrated to a small volume by rotary evaporation. Ice was added, and after 2 days, brown crystals had formed. These were collected by filtration (62 mg), and a m.p. of 125-127°C was observed (literature 128-130°C, for crude product). A solution of these brown crystals was derivatizated with diazomethane and analyzed by GC-mass spectrometry (GC-MS). The major peak had a molecular ion of 249, equal to the mass of the methylated first product, 4-(2-formamidobenzoyl)butanoic acid.

In the second reaction step, excess concentrated HCl was added to the brown crystals and the mixture was heated on a steam bath for 30 min. The reaction solution was then diluted with H_2O and extracted with ethyl acetate. The organic extract was concentrated, reacted with diazomethane, and analyzed by GC-MS to verify the formation of methylated 5-(2-aminophenyl)-5-oxo-pentanoic acid.

Several attempts to synthesize *cis, cis*-muconic acid by reacting phenol with 13.5% peracetic acid (Elvidge *et al*., 1950) were unsuccessful.

5.2.13 Analytical methods

HPLC analyses were done using a HP series 1050 instrument equipped with a variable wavelength detector. A reverse phase column was used (LiChrospher 100 RP-18, 125 x 4 mm column with 5 µm diameter packing material, HP) and supernatant extracts or aqueous culture samples were analyzed using an isocratic mobile phase of 1% aqueous acetic acid and methanol at a flow rate of 1.0 mL/min. For carbazole and anthranilic acid analyses the effluent was monitored at 254 nm and the mobile phase was a 50:50 mixture of the two solvents. An 80:20 (1% aqueous acetic acid:methanol) mixture was used for catechol analysis and the wavelength used was 280 nm.

GC analyses were performed on a HP model 5730A gas chromatograph equipped with a FID to quantitate loss of carbazole or other substrates and to analyze BSA-derivatized samples. Extracts from cultures that contained a sulfur-heterocycle were also analyzed by this GC with a sulfur-selective flame photometric detector (Fedorak and Grbic´-Galic´, 1991). Peak areas were obtained using an HP model 3390A integrator. A Varian model 3600 gas chromatograph equipped with a FID and a nitrogen-selective detector was used to detect carbazole metabolites and other nitrogen-containing compounds. Varian GC Star Workstation Software was used to handle data from this GC. A 30 m DB-5 capillary column was used for all GC analyses, except for one experiment which used a DB-1 capillary column. The temperature program routinely used was 90°C for 2 min, increasing by 4°C per min to 250°C then held for 2 min.

Selected extracts were analyzed by electron impact and chemical ionization (using ammonia as the reagent gas) (GC-MS at the Mass Spectrometry Laboratory in the Chemistry Department at the University of Alberta (Fedorak and Westlake, 1986). GC-FTIR (GC-Fourier transform infrared spectroscopy) analysis was performed at the Spectral Services Laboratory in the Chemistry Department at the University of Alberta.

Liquid scintillation counting was done with a Beckman Model LS 3801 liquid scintillation counter. A Philips Model PU 8740 UV/Vis scanning spectrophotometer was used for enzyme assays (260, 375 nm) and some growth measurements (600 nm).

Purified carbazole metabolites (compounds **VI** and **X**) were dissolved in $CDCl_3$ and analyzed using NMR (nuclear magnetic resonance) techniques. The predicted structure of compound **VI** was verified using one- and two-dimensional ^1H NMR. Gradient-

Enhanced Correlation (GCOSY), Heteronuclear Multiple Bond Coherence (HMBC), Heteronuclear Multiple Quantum Coherence (HMQC), and APT (Attached Proton Test) experiments were conducted to help eludicate the structure of compound **X**. All NMR experiments were carried out on a Varian Unity 500 spectrometer by Dr. Albin Otter in the Department of Chemistry at the University of Alberta. More detailed descriptions of the NMR experiments conducted have been written by Dr. A. Otter and are included in Appendix B.

5.3 RESULTS

5.3.1 Identification of isolate LD2

Isolate LD2 grew in mineral medium that contained carbazole as the sole source of carbon, nitrogen, and energy. To ensure that it was not fixing N_2, an acetylene reduction assay was performed which showed no nitrogenase activity when the isolate was grown in B-N medium with carbazole. Thus this strain used carbazole as its nitrogen source, as well as its carbon and energy source. This organism, designated isolate LD2, was identified as a *Pseudomonas* species by the following characteristics: Gram negative, motile rod; oxidase and catalase positive; acid produced aerobically on oxidative-fermentative medium with glucose; no anaerobic growth; and indole, methyl red, and Voges-Proskauer negative. This isolate was found to most closely resemble *Pseudomonas fluorescens* on the basis of additional biochemical tests conducted in the Microbiology Section of the Alberta Environmental Centre (Vegreville, AB). The isolate grew well on glucose, acetate, pyruvate, and succinate in B+N medium.

5.3.2 Growth on and loss of carbazole

Pseudomonas sp. LD2 was inoculated into several flasks containing B-N medium and carbazole, and at various times a culture was analyzed for bacterial numbers by plate counts and one of the replicate cultures was acidified and extracted in order to examine the degradation of carbazole over time. Figure 5.1 shows the growth of isolate LD2 with a concommitant loss of carbazole. Within 24 h, isolate LD2 reached stationary phase and had degraded 87% of the carbazole, compared with a sterile control. By 96 h of incubation, carbazole was no longer detected by GC. When isolate LD2 was grown in B+N medium and compared to growth in B-N medium, no significant difference was observed in the extent of carbazole degradation, although degradation proceeded slightly faster when no inorganic nitrogen source was supplied. Carbazole degradation by isolate LD2 proceeded at similar rates in a nutrient-rich medium (TSB) or in B-N medium. Furthermore, isolate LD2 retained its carbazole-degrading ability after several successive transfers in TSB medium.

Growth experiments with the mixed culture CM showed a 10-fold increase in bacterial numbers after a lag of 4 days. Stationary phase was reached by 10 days of incubation, corresponding to a complete loss of carbazole from the medium as determined by GC. For the DC culture, bacterial numbers increased 10-fold within 24 h, and only about 15% of the added carbazole remained in the medium after 3 days of incubation. Despite this, complete carbazole degradation was not evident in this culture until after 10 days. The bacterial numbers in the DC culture always decreased with prolonged incubation which corresponded with the appearance of "clumps" of bacteria in the liquid medium. No growth experiments with the D5 culture were conducted, although this culture became more turbid in the presence of carbazole than the CM or DC cultures.

5.3.3 Carbon and nitrogen limitation in isolate LD2

To determine whether isolate LD2 was carbon- or nitrogen-limited at the end of its growth phase, this carbazole-degrading bacterium was incubated in B-N medium with carbazole as the sole carbon and nitrogen source. After the culture reached stationary phase, portions of the culture were supplemented with glucose, carbazole, NH_4NO_3 (at

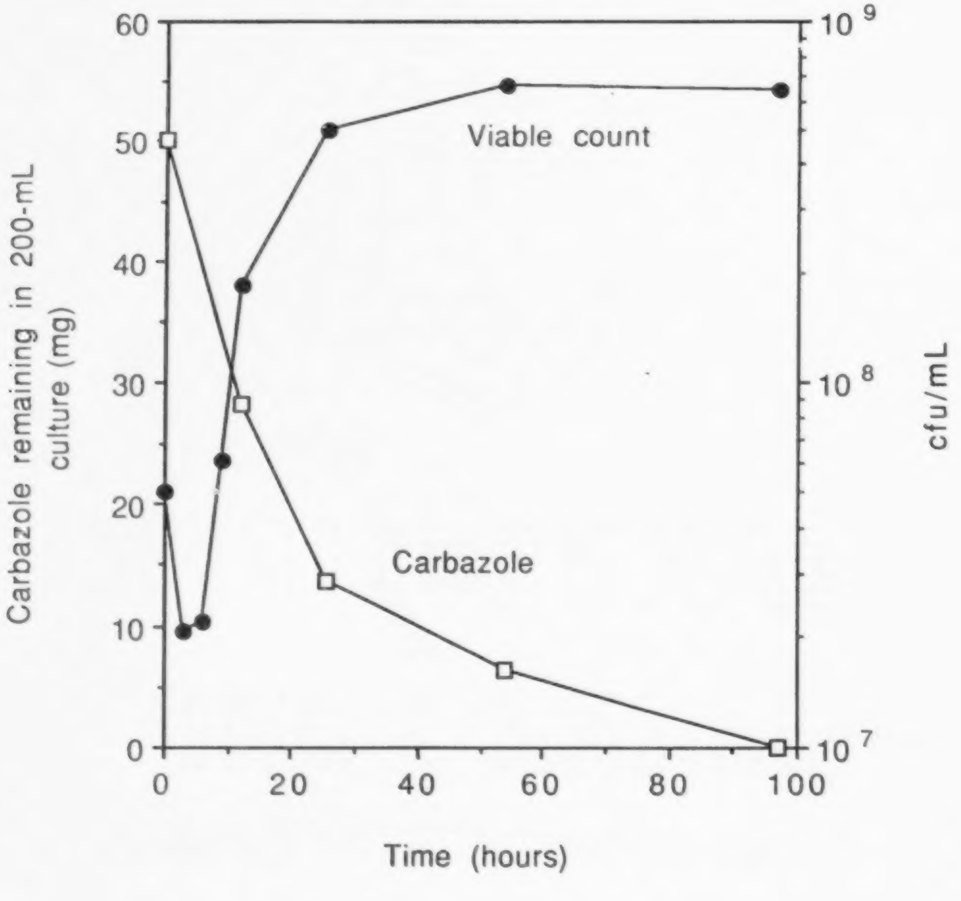


Figure 5.1 Growth of isolate LD2 in liquid medium with carbazole as the sole source of carbon, nitrogen, and energy.

the same concentration as in B+N medium), or with trace NH_4NO_3, and an unamended portion served as a control. Growth was monitored by measuring OD_{600}. After 3 days of incubation, only the glucose- and carbazole-supplemented cultures grew, indicating that carbon was limiting at the end of the growth phase of the initial culture.

5.3.4 Mineralization experiments

5.3.4.1 Carbazole mineralization

When isolate LD2 was grown in B-N medium with [U-^{14}C]carbazole, 43% of the radioactivity was released as $^{14}CO_2$ during the first 3 days of incubation. After 7 days of incubation, 57% of the added radioactivity was recovered as $^{14}CO_2$, with 70% recovered after 30 days. A comparison of the carbazole mineralization rates of LD2 and the mixed cultures D5, CM, and DC is shown in Fig. 5.2. Similar rates of carbazole mineralization were observed for LD2, D5, and DC, with close to 40% of the added radioactivity released as $^{14}CO_2$ in these cultures after 3 days of incubation. There was a lag of about 5 days before carbazole mineralization was observed in the CM culture, in agreement with the growth data above. After 14 days of incubation, close to 70% $^{14}CO_2$ was recovered in the LD2 and D5 cultures, with 55% and 57% $^{14}CO_2$ recovered in the DC and CM cultures, respectively.

5.3.4.2 Mineralization of carbazole, glucose, and acetate

Isolate LD2 was inoculated into flasks of B-N medium containing glucose or acetate as the major carbon source and carbazole as the sole nitrogen source. This experiment was designed to see if isolate LD2 was able to extract the nitrogen atom from the carbazole molecule to serve as its nitrogen source while growing on simple carbon sources, possibly leaving a biphenyl intermediate in a manner similar to the '4S' pathway of dibenzothiophene degradation (Krawiec, 1990). When glucose was added as the major

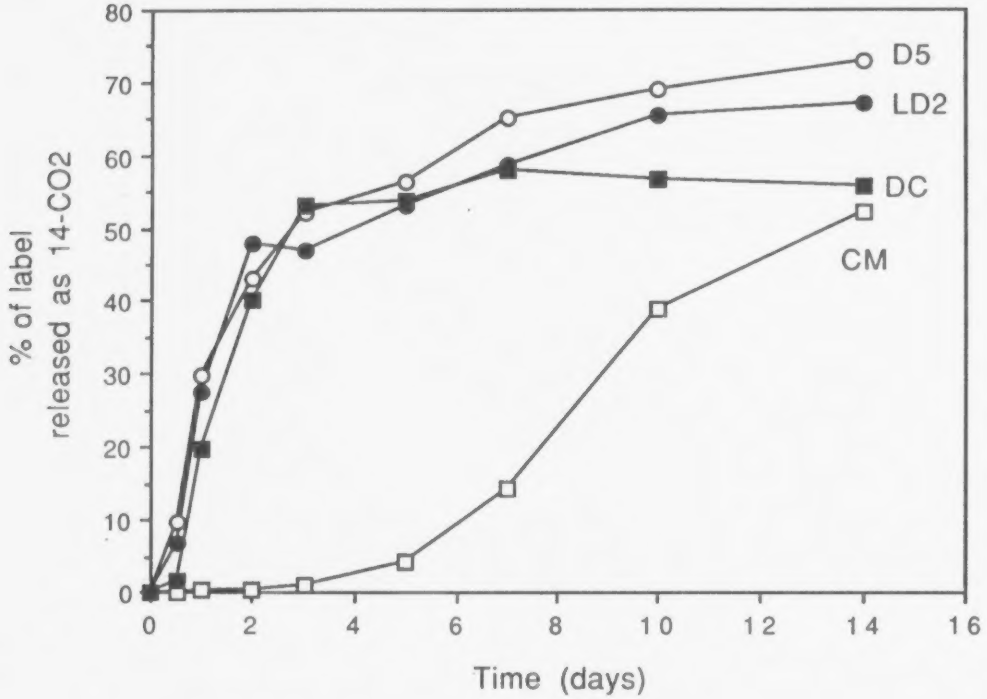


Figure 5.2 Mineralization of ^{14}C-carbazole by isolate LD2 and by the mixed carbazole-degrading cultures CM, DC, and D5.

carbon source in B-N medium, carbazole was preferentially mineralized, although the presence of glucose slowed carbazole mineralization slightly (Fig. 5.3a). Similarly, when acetate was added as a carbon source and carbazole was added as a nitrogen source, carbazole was preferentially mineralized (Fig. 5.3b). The same trends were observed for both substrates when the experiments were carried out in B+N medium in which ammonium and nitrate were present as nitrogen sources in addition to carbazole (data not shown).

5.3.4.3 Mineralization of labelled compounds in Norman Wells crude oil

Environmental isolates often have the ability to oxidize and/or degrade a wide range of aromatic hydrocarbons and heterocyclic compounds (Foght and Westlake, 1988; Strubel *et al.*, 1989; Boldrin *et al.*, 1993; Monna *et al.*, 1993). To see if isolate LD2 could mineralize different hydrocarbons, replicate cultures were incubated with Norman Wells crude oil containing one of five ^{14}C-labelled compounds. After 28 days of incubation, the proportion of ^{14}C recovered as $^{14}CO_2$ from the various compounds were: biphenyl, 1.3%; naphthalene, 0.8%; phenanthrene, 0.1%; anthracene, 0.7%; and hexadecane, 4.2%. The positive control containing carbazole released 42% of the radioactivity as $^{14}CO_2$. Using the criterion that significant mineralization occurred when greater than 5% of the label from ^{14}C-labelled compounds was recovered as $^{14}CO_2$ (Foght *et al.*, 1990) isolate LD2 did not mineralize any of the tested hydrocarbons, although carbazole mineralization was observed in the presence of crude oil.

5.3.5 Metabolism of other aromatic compounds by isolate LD2

Several aromatic compounds were tested as growth substrates for isolate LD2 and some of these compounds were included in cultures of isolate LD2 growing on carbazole to see if they could be biotransformed. Figure 5.4 gives the structures of some of these compounds. Of the 20 aromatic compounds listed in Table 5.2, isolate LD2 only grew on

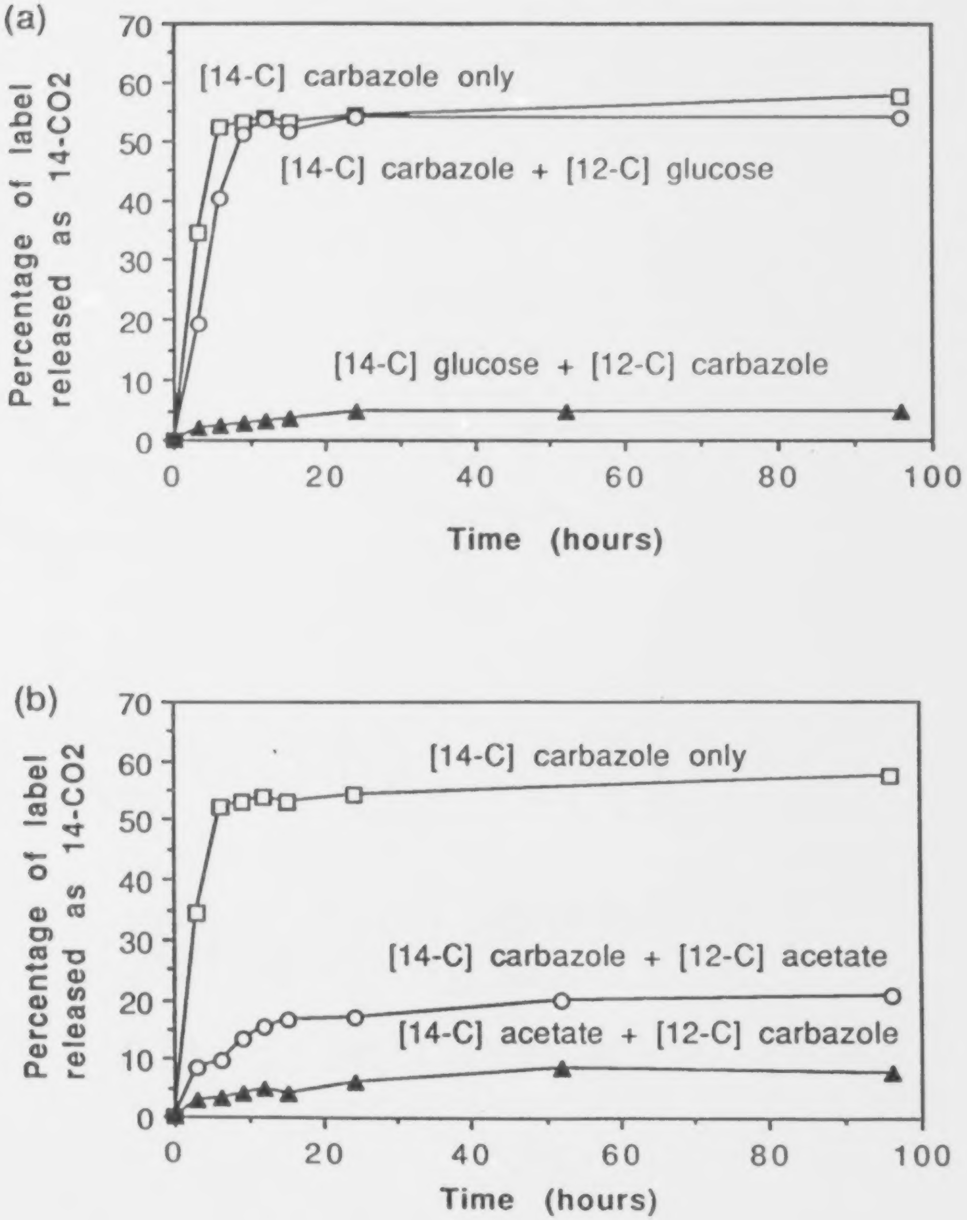
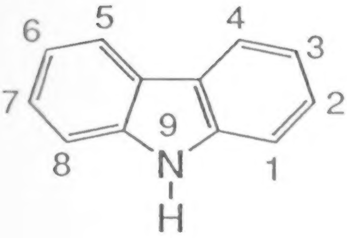


Figure 5.3 Mineralization of (a) ^{14}C-carbazole and ^{14}C-glucose and (b) ^{14}C-carbazole and ^{14}C-acetate by isolate LD2 when these compounds were supplied as co-substrates in B-N medium.


6
5
4
3
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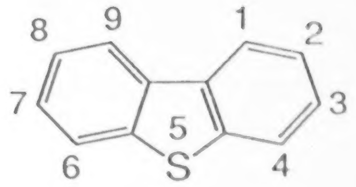
1
H

Carbazole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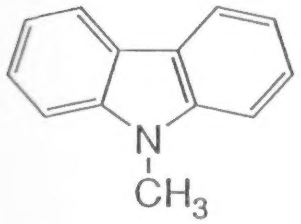
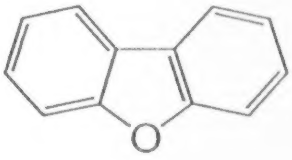
S
4

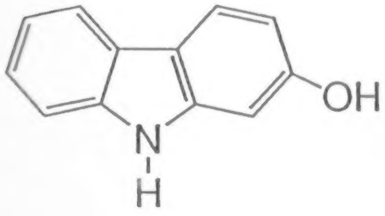
Dibenzothiophene



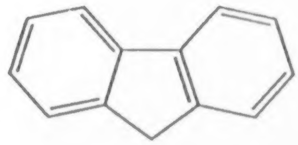
9-Methylcarbazole



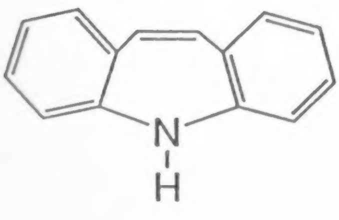
Dibenzofuran



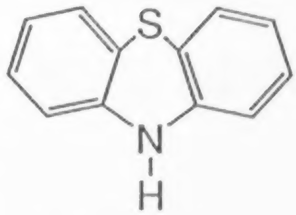
2-Hydroxycarbazole



Fluorene



Iminostilbene



Phenothiazine

Figure 5.4 Structures of some aromatic compounds tested as growth substrates for isolate LD2.

Table 5.2 Aromatic compounds tested as growth substrates for *Pseudomonas* sp. LD2

Compound	Supported growth of strain LD2[a]	Transformed by strain LD2 in the presence of carbazole
Benzene	No	ND[b]
Naphthalene	No	ND
Phenanthrene	No	ND
Fluorene	No	Yes
Dibenzofuran	No	Yes
Dibenzothiophene	No	Yes
Phenothiazine	No	Yes
Iminostilbene	No	Yes
9-Methylcarbazole	No	No
2-Hydroxycarbazole	No	No
1,2,3,4-Tetrahydrocarbazole	No	Yes
Biphenyl	No	ND
2,2'-Dihydroxybiphenyl	No	ND
2,3-Dihydroxybiphenyl	Yes	ND
2-Aminobiphenyl	No	No
2-Nitrobiphenyl	No	No
Benzoic acid	No	ND
Salicylic acid	No	ND
Gentisic acid	No	ND
4-Hydroxybenzoic acid	Yes	ND

[a] Isolate LD2 was incubated in B-N medium unless the test compound did not contain a N atom in its molecular structure, then B+N was used as the growth medium

[b] ND=not determined

4-hydroxybenzoic acid and 2,3-dihydroxybiphenyl. It did not grow on naphthalene or phenanthrene, in agreement with its inability to mineralize these compounds in the presence of Norman Wells crude oil.

5.3.5.1 Carbazole analogs

None of the carbazole analogs, dibenzothiophene, fluorene or dibenzofuran supported the growth of isolate LD2 in B+N medium. However, when these analogs were incubated with isolate LD2 in the presence of carbazole in B-N medium, they were biodegraded to some extent, and transformation products were detected (Table 5.2). Table 5.3 shows that carbazole was degraded in the presence of these analogs, and that the concentration of each analog decreased in the presence of carbazole. Growth experiments with carbazole and one of the three analogs showed a decrease in the viable cell numbers of isolate LD2 with prolonged incubation (Fig. 5.5). The presence of dibenzofuran had the most inhibitory effect. Cell death in the presence of carbazole analogs was thought to result from the accumulation of analog metabolites which are toxic to isolate LD2.

In order to deduce what these metabolites might be, acid extracts of LD2 cultures incubated with carbazole and one of the three analogs were treated with diazomethane and analyzed by GC-MS. The transformation product of dibenzothiophene was identified as dibenzothiophene sulfoxide by comparison of the GC retention time and mass spectrum of the metabolite with those of an authentic sample of the sulfoxide, and the mass spectrum is shown in Fig. 5.6a. No new metabolites were observed when acidified extracts of carbazole and dibenzothiophene cultures were derivatized with diazomethane.

9-Fluorenone and 9-fluorenol were identified as biotransformation products of fluorene when this compound was present in culture medium with carbazole. These compounds were identified based on comparison of their mass spectra with those reported in the literature (Grifoll *et al.*, 1992; Grifoll *et al.*, 1994) and the mass spectra of these compounds are shown in Fig. 5.6b and c. No other fluorene metabolites reported by

Table 5.3 Loss of carbazole and its analogs in LD2 cultures incubated for 7 days.

	LOSS OF SUBSTRATE (%)[a]	
SUBSTRATE(S)	CARBAZOLE	ANALOG
Carbazole	100	NA[b]
Carbazole + Dibenzothiophene	100	50
Carbazole + Dibenzofuran	92	44
Carbazole + Fluorene	99	17

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[a] loss relative to sterile control; calculated from GC peak areas of neutral extracts

[b] NA = not applicable

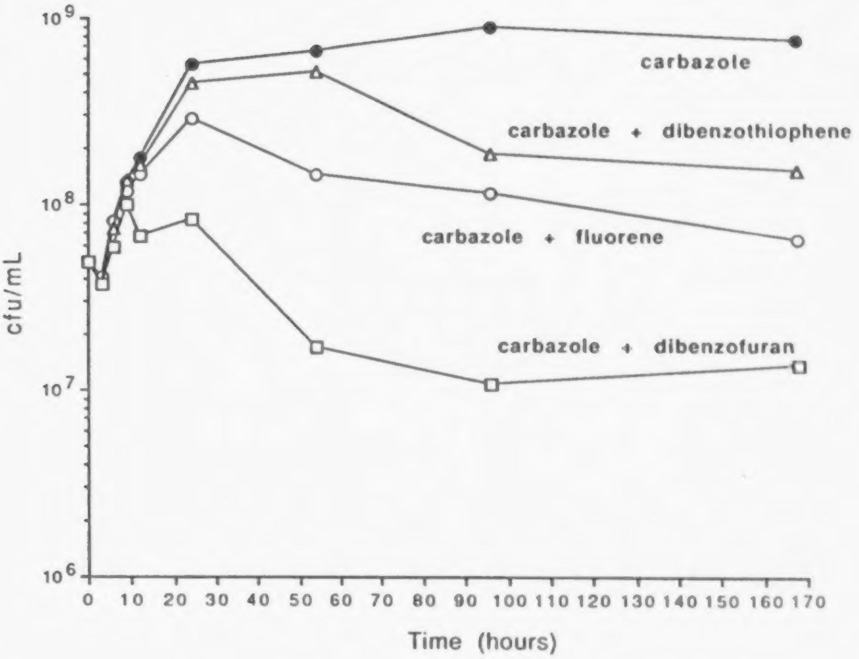


Figure 5.5 Viable cell counts of isolate LD2 incubated with carbazole or carbazole and one of dibenzothiophene, dibenzofuran, or fluorene.

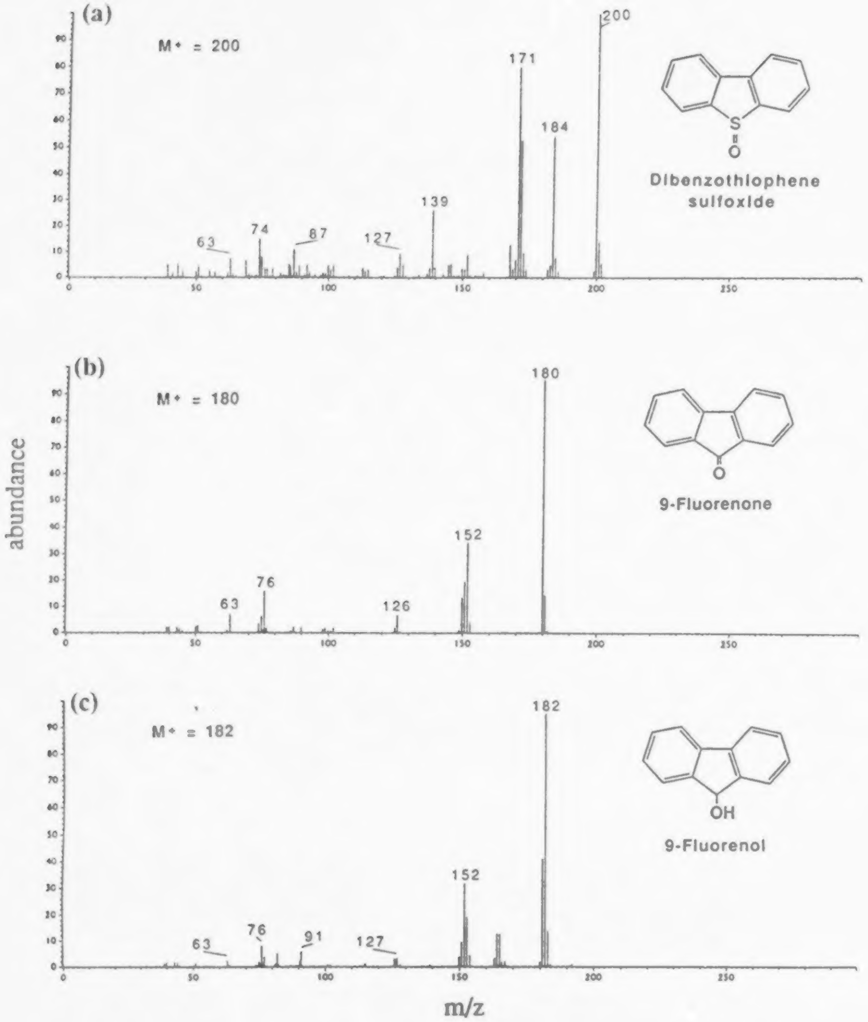


Figure 5.6 Mass spectra of analog transformation products detected by GC-MS when isolate LD2 was incubated with carbazole and dibenzothiophene (a) or carbazole and fluorene (b and c).

Grifoll *et al*. (1994) were detected.

When acidified supernatant extracts of isolate LD2 incubated with carbazole and dibenzofuran were derivatized with diazomethane, numerous dibenzofuran metabolites were detected and were identified by comparing their mass spectra and molecular formulae with those reported in the literature (Fortnagel *et al*., 1990; Strubel *et al*., 1991). The mass spectra and predicted fragmentation patterns of these compounds are shown in Fig. 5.7. The metabolites, identified as their methyl esters, included salicylic acid (M^{+}= 152), 3-(chroman-4-on-2-yl)acetic acid (M^{+}= 220), 3-(chroman-4-on-2-yl)pyruvic acid (M^{+}= 248), and 3-(chroman-4-on-2-yl)lactic acid (M^{+}= 250). In addition, two N-containing metabolites accumulated in LD2 cultures incubated with carbazole and dibenzofuran. These are discussed further in Section 5.3.7.2.3.

5.3.5.2 Other aromatic compounds

When phenothiazine, iminostilbene, or 1,2,3,4-tetrahydrocarbazole was present in carbazole-containing medium, no transformation products were observed by GC analysis. However, the amounts of phenothiazine, iminostilbene, and 1,2,3,4-tetrahydrocarbazole were reduced by 73%, 25%, and 61%, respectively compared to sterile controls after 2 weeks of incubation. Carbazole was completely degraded in the presence of iminostilbene, and degraded by about 90% when phenothiazine or 1,2,3,4-tetrahydrocarbazole was present in the medium.

Neither 9-methylcarbazole nor 2-hydroxycarbazole was degraded or biotransformed by isolate LD2. When carbazole was present in cultures with these compounds, no decreases in the amounts of these compounds were seen, and no transformation products were observed by GC. Carbazole was completely degraded in the presence of 9-methylcarbazole, but of the 10 compounds tested in the presence of carbazole, only 2-hydroxycarbazole inhibited carbazole degradation. The methyl group in the 9 position and hydroxyl group in the 2 position appeared to interfere with the initial oxidations of the

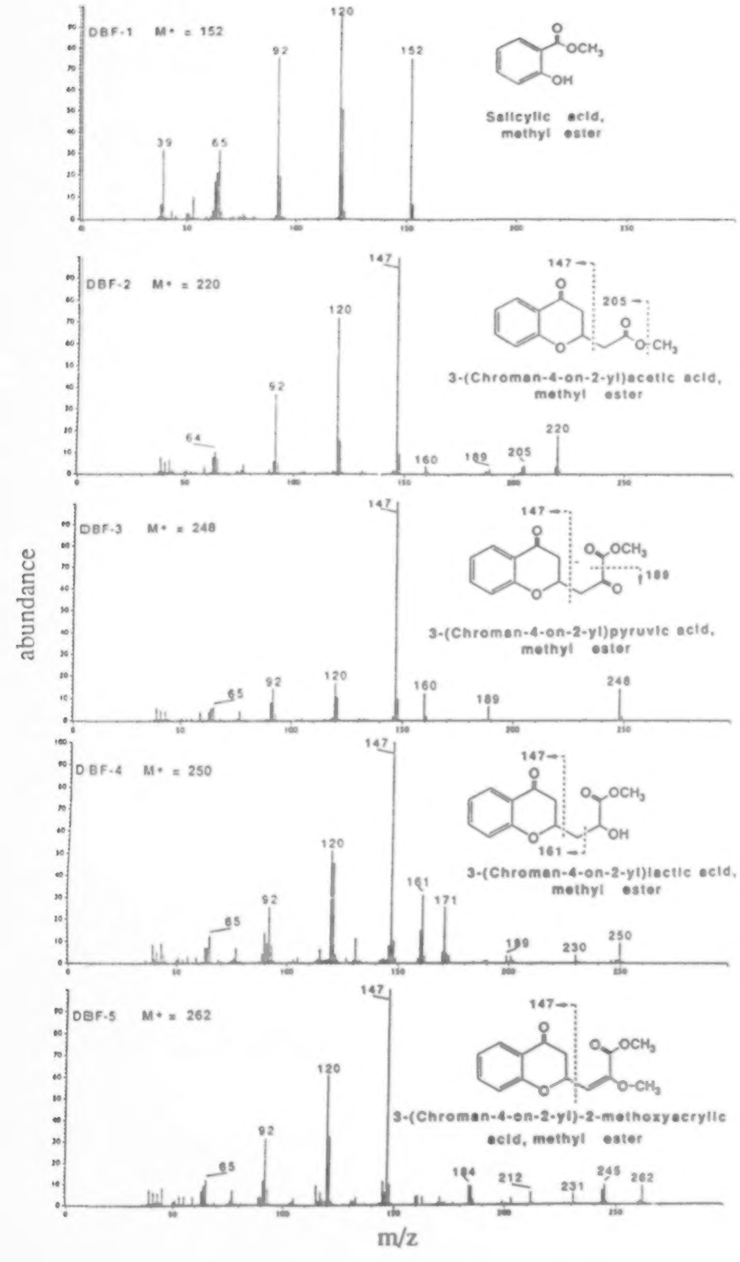


Figure 5.7 Mass spectra of dibenzofuran degradation products detected in culture supernatants when isolate LD2 was incubated with carbazole and dibenzofuran.

carbazole molecule by isolate LD2, suggesting that the oxidations occurred near or at the nitrogen atom.

Biphenyl and its derivatives 2,2'-dihydroxybiphenyl, 2-nitrobiphenyl, and 2-aminobiphenyl did not serve as growth substrates for isolate LD2. 2-Nitrobiphenyl and 2-aminobiphenyl were not degraded nor biotransformed in the presence of carbazole, although carbazole was completely degraded. If isolate LD2 degraded carbazole by removing the nitrogen atom in a manner analogous to the '4S' pathway for dibenzothiophene metabolism whereby the sulfur atom is selectively removed (Krawiec, 1990) one of these biphenyls might be an intermediate. However, because none of these supported the growth of isolate LD2, and none were observed in extracts of LD2 cultures incubated with carbazole, direct attack of the pyrrole ring is not likely the mechanism of carbazole degradation.

5.3.6 Growth on anthranilic acid and catechol

Anthranilic acid and catechol were reported as metabolites resulting from the aerobic bacterial degradation of carbazole (Ouchiyama *et al.*, 1993; Resnick and Gibson, 1993; Hisatsuka and Sato, 1994). These two compounds were tested as growth substrates for *Pseudomonas* sp. LD2. Growth of isolate LD2 on anthranilic acid (250 mg/L) and its depletion from B-N medium were observed (Fig. 5.8). The culture reached stationary phase by 16 h of incubation, and at that time, anthranilic acid was no longer detected in the aqueous medium by HPLC. A single metabolite (denoted compound **Q**) was detected by HPLC after the first 4 h of incubation. When anthranilic acid-grown cultures were acidifed, extracted with ethyl acetate, and reacted with BSA, a metabolite eluting at the same time as BSA-derivatized catechol was detected by GC. The presence of catechol as a metabolite from anthranilic acid degradation by isolate LD2 was verified by TLC. Isolate LD2 was also able to grow on catechol (250 mg/L) as a carbon source in B+N medium, and by 23 h, catechol was depleted from the culture as detected by HPLC. The formation

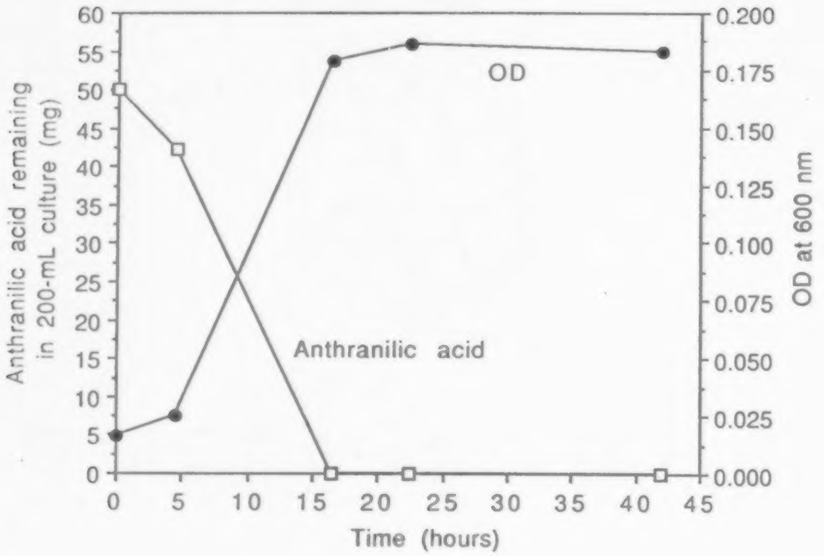


Figure 5.8 Growth of isolate LD2 on anthranilic acid and the loss of this compound in B-N medium.

of a metabolite from catechol was observed by HPLC, and it had the same HPLC retention time as compound **Q**.

A time course experiment was done to compare the rates of carbazole and anthranilic acid utilization by isolate LD2 from a mixture of these two compounds using either carbazole-grown cells or anthranilic acid-grown cells as the inoculum. Growth measurements (OD_{600}) showed that the anthranilic acid-grown inoculum grew at a slightly faster rate on these two compounds than the carbazole-grown inoculum, but both cultures reached stationary phase after 48 h of incubation. Although anthranilic acid is far more water-soluble than carbazole, both of these substrates were used simultaneously by these cultures (Fig. 5.9). Anthranilic acid was depleted from the medium within 24 h, regardless of inoculum, whereas carbazole was still detected after 48 h.

To determine whether the enzymes catalyzing carbazole and anthranilic acid degradation by isolate LD2 were inducible or constitutive, the organism was grown on glucose, anthranilic acid, or carbazole to early stationary phase and washed cells were added to fresh medium containing carbazole or anthranilic acid in the presence or absence of CAP, an inhibitor of protein synthesis. Cells grown with carbazole degraded anthranilic acid by 3 h of incubation, even when CAP was present (Fig. 5.10). Similarly, anthranilic acid-grown cells degraded anthranilic acid by 4 h in the absence of CAP and by 9 h when CAP was present. The concentration of anthranilic acid in the medium decreased after a lag of 5 hours by glucose-grown cells in the absence of CAP, but did not decrease in concentration when CAP was present (Fig. 5.10). Even after 60 h of incubation, the concentration of anthranilic acid did not decrease when glucose-grown cells were incubated with CAP. A similar experiment showed that anthranilic acid-grown cells consumed carbazole in the presence or absence of CAP. These results suggested that the enzymes for carbazole and anthranilic acid degradation were inducible in isolate LD2, and that anthranilic acid can serve as an inducer for carbazole degradation. Anthranilic acid can also induce the naphthalene oxidative pathway in *Pseudomonas putida* (Yen and Serdar, 1988).

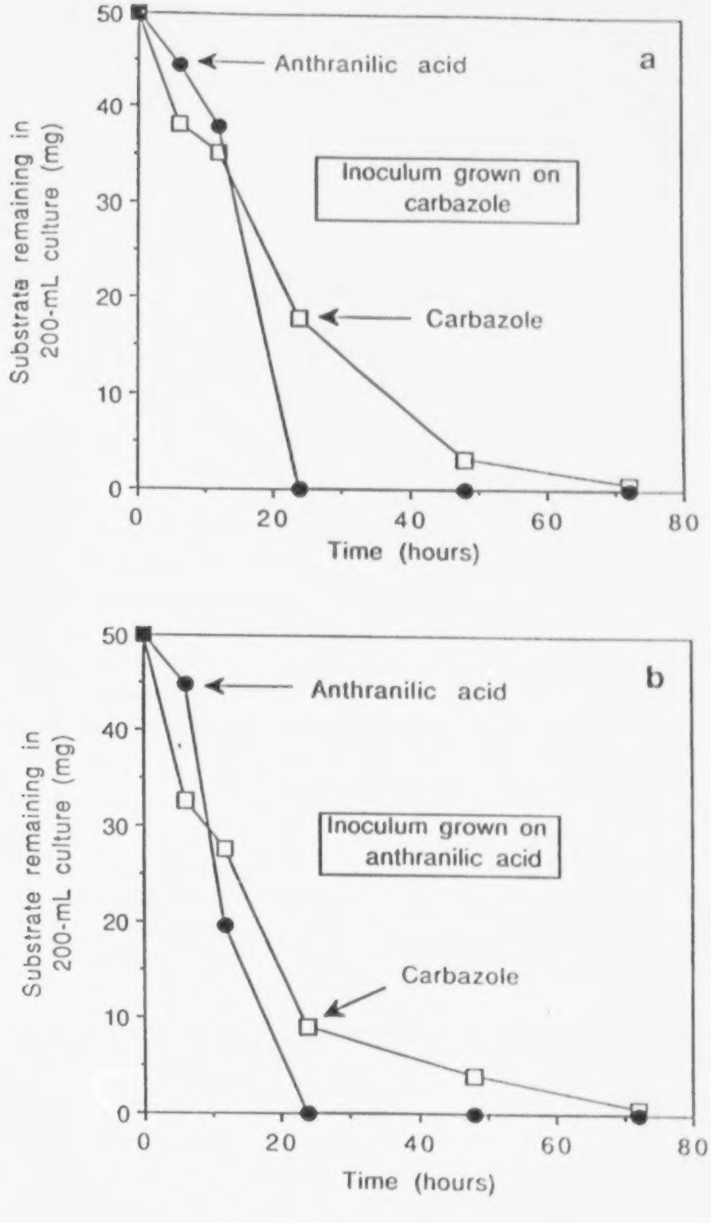


Figure 5.9 Loss of carbazole and anthranilic acid when a mixture of these compounds was supplied and incubated with either (a) carbazole-grown or (b) anthranilic acid-grown LD2 cells.

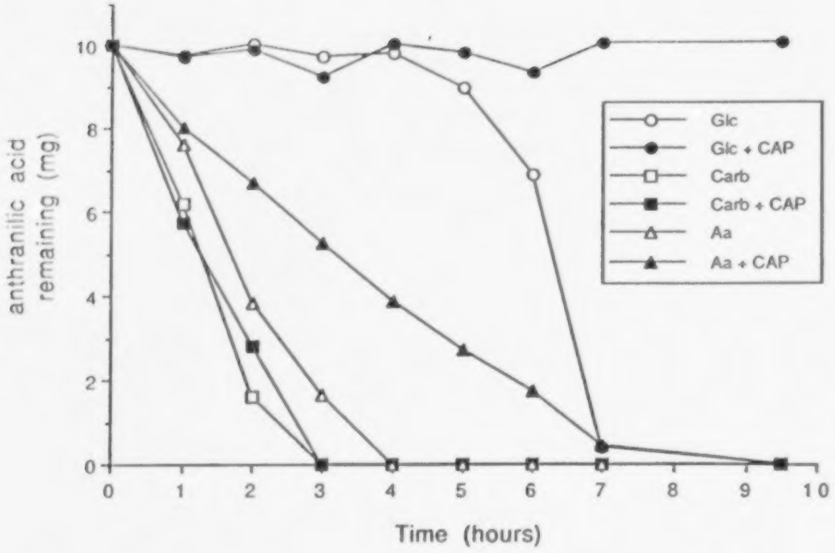


Figure 5.10 Loss of anthranilic acid by glucose- (Glc), anthranilic acid- (Aa) or carbazole-grown (Carb) LD2 cells in the presence or absence of CAP.

5.3.7 Identification of carbazole metabolites

5.3.7.1 BSA-treated LD2 culture extracts

Because anthranilic acid and catechol were found to be substrates for the growth of isolate LD2, cultures grown on carbazole were examined for the presence of these compounds in a washed suspension of carbazole-grown LD2 cells incubated with carbazole for 3 or 7 h. The cells were then broken by sonication, extracted under neutral and acidic conditions with ethyl acetate, and analyzed by HPLC. The 7 h acidic extract chromatogram showed the presence of a metabolite which eluted at the same time as authentic anthranilic acid. This extract was derivatized with BSA and analyzed by GC-MS. A metabolite in the culture extract had the same GC retention time and mass spectrum as a BSA-derivatized anthranilic acid standard (M^{+}= 281). Catechol was not detected using this method.

For the washed cell experiments above, isolate LD2 was incubated for 40 h with carbazole before being harvested. The supernatant resulting after centrifugation of the cultures was saved and subsequently extracted with ethyl acetate under neutral and acidic conditions. In the acidified supernatant extract, one large metabolite peak was seen by GC after derivatization with BSA. GC-high-resolution MS gave a molecular formula of $C_{12}H_{22}O_4Si_2$ (M^{+}= 286.10512) for this compound which matched that of BSA-derivatized 2,4-hexadienedioic acid (muconic acid) from the GC-MS computer library. This compound was also present in BSA-derivatized extracts of anthranilic acid- and catechol-grown cells, presumably the BSA-derivative of compound **Q**. *cis, cis*-Muconic acid was believed to be the isomer formed because this peak did not match the GC retention time of *trans, trans*-muconic acid. Furthermore, isolate LD2 was found to have catechol 1,2-dioxygenase activity (specific activity = 0.30 µmol/min/mg protein), in which *cis, cis*-muconic acid is the *ortho* cleavage product of catechol. HPLC analysis of the catechol 1,2-dioxygenase reaction mixture sampled at different times showed the formation of a peak eluting immediately before catechol which increased in size over time. This product, presumably *cis, cis*-muconic acid, had the same GC retention time as compound **Q**, thus verifying that *cis, cis*-muconic acid is indeed compound **Q**. Catechol 2,3-dioxygenase activity was not detected in isolate LD2.

Thus, using BSA as a derivatizing agent, anthranilic acid, catechol, and *cis, cis*-muconic acid were identified as carbazole metabolites. It was difficult to identify other carbazole metabolites in extracts derivatized with BSA because the nitrogen-selective detector used for GC analysis was insensitive to nitrogen in the presence of silicone. Thus, diazomethane was used to replace BSA as a derivatizing agent. The following sections describe the carbazole metabolites identified after treatment of LD2 extracts with diazomethane.

5.3.7.2 Diazomethane-treated LD2 culture extracts

Other metabolites resulting from carbazole degradation by isolate LD2 were detected by GC after derivatization of acidified supernatant extracts with an ethereal solution of diazomethane. Using this derivatization procedure, methyl esters were formed from carboxylic acids (except for one metabolite, in which a methyl ether was formed). A portion of the total ion current chromatogram of a diazomethane-treated acidified culture extract is shown in Fig. 5.11. When this sample was analyzed using a nitrogen-selective detector, 10 nitrogen-containing metabolites (labelled with Roman numerals in Fig. 5.11) were detected. None of these was present in the corresponding sterile control. The peak eluting between compounds **VI** and **VII** is a phthalate, a common impurity in GC and GC-MS analyses (McLafferty, 1980).

Figures 5.12 to 5.16 show the mass spectra of the 10 compounds labelled in Fig. 5.11. The spectra are presented in the order that they are discussed in the text. A compound corresponding to the dimethyl ester of *cis, cis*-muconic acid (M^+= 170.05791) was also found in this sample (spectrum not shown). The proposed structure of each metabolite is shown (Figs. 5.12 to 5.16) with each spectrum (except for compounds **IV**

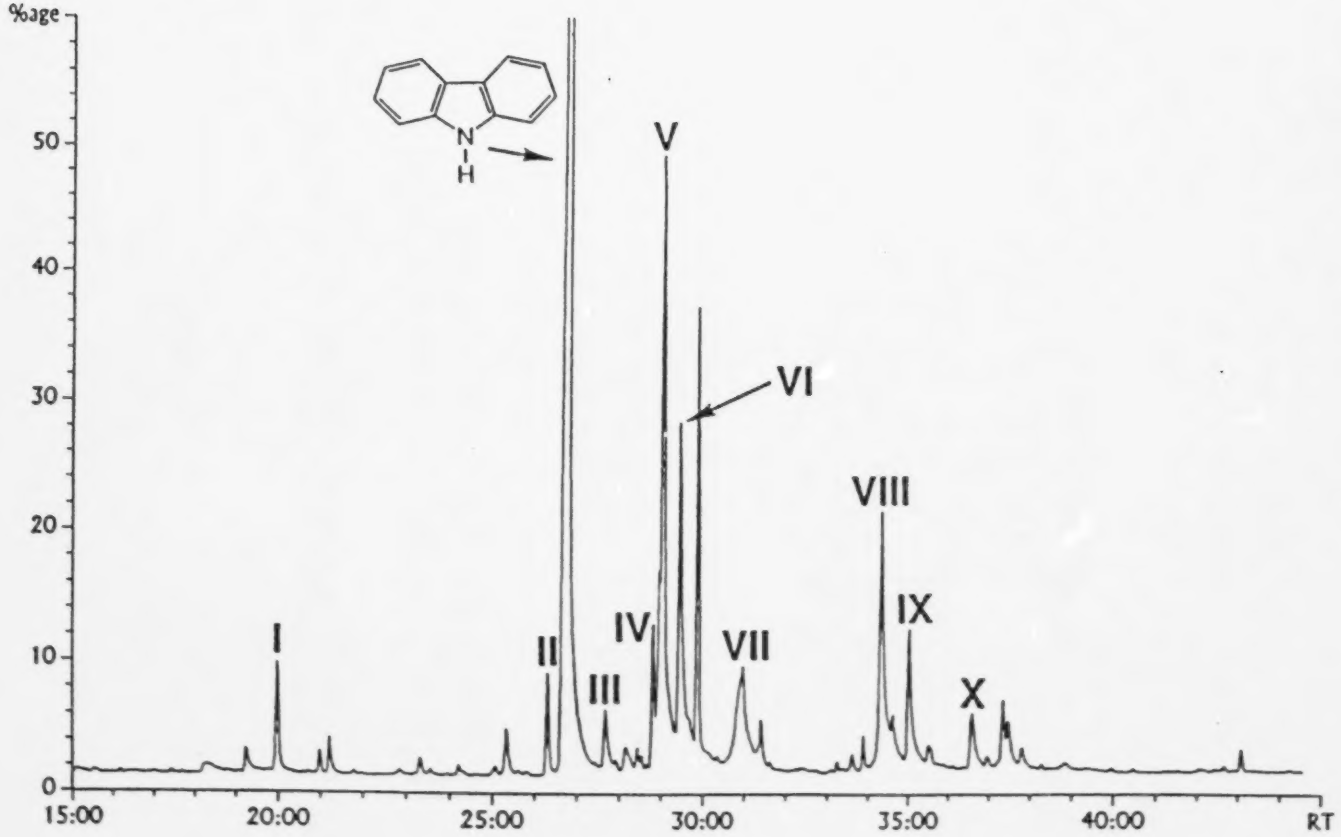


Figure 5.11 A portion of the total ion current chromatogram from the GC-MS analysis of a diazomethane-treated extract from a culture of isolate LD2 grown on carbazole. Each of the labelled peaks is a nitrogen-containing metabolite.

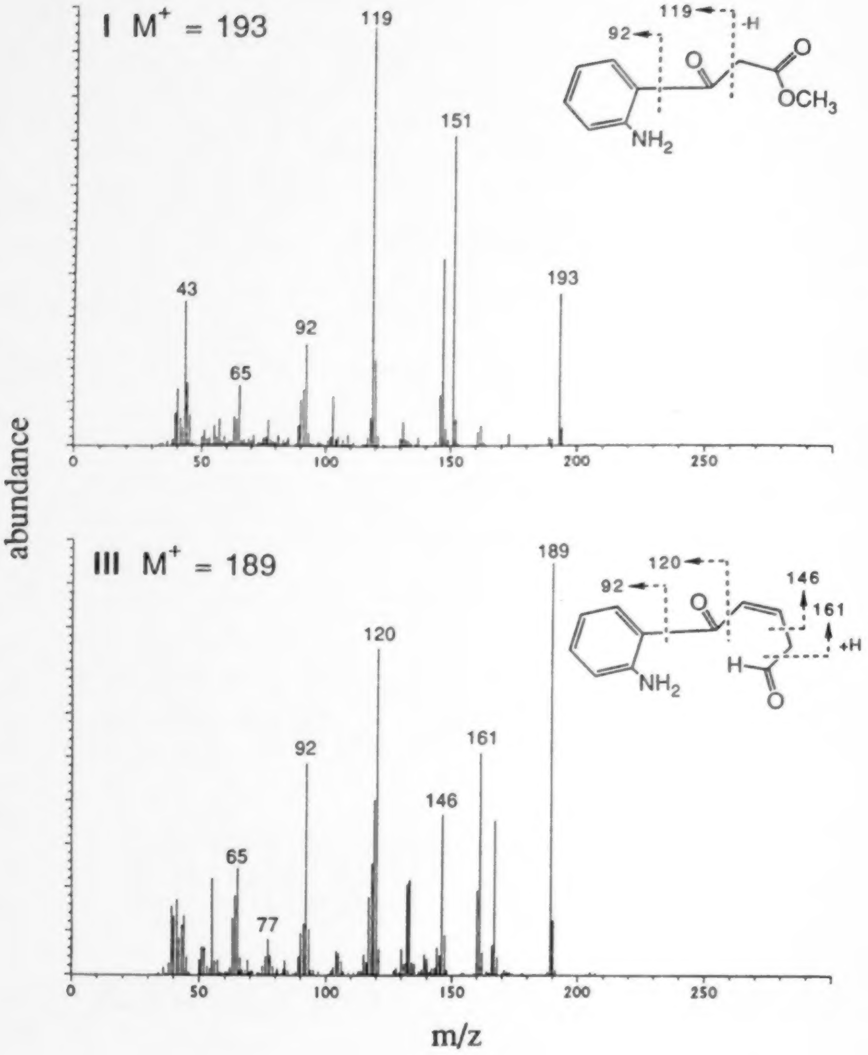


Figure 5.12 The mass spectra and proposed structures of compounds **I** and **III**.

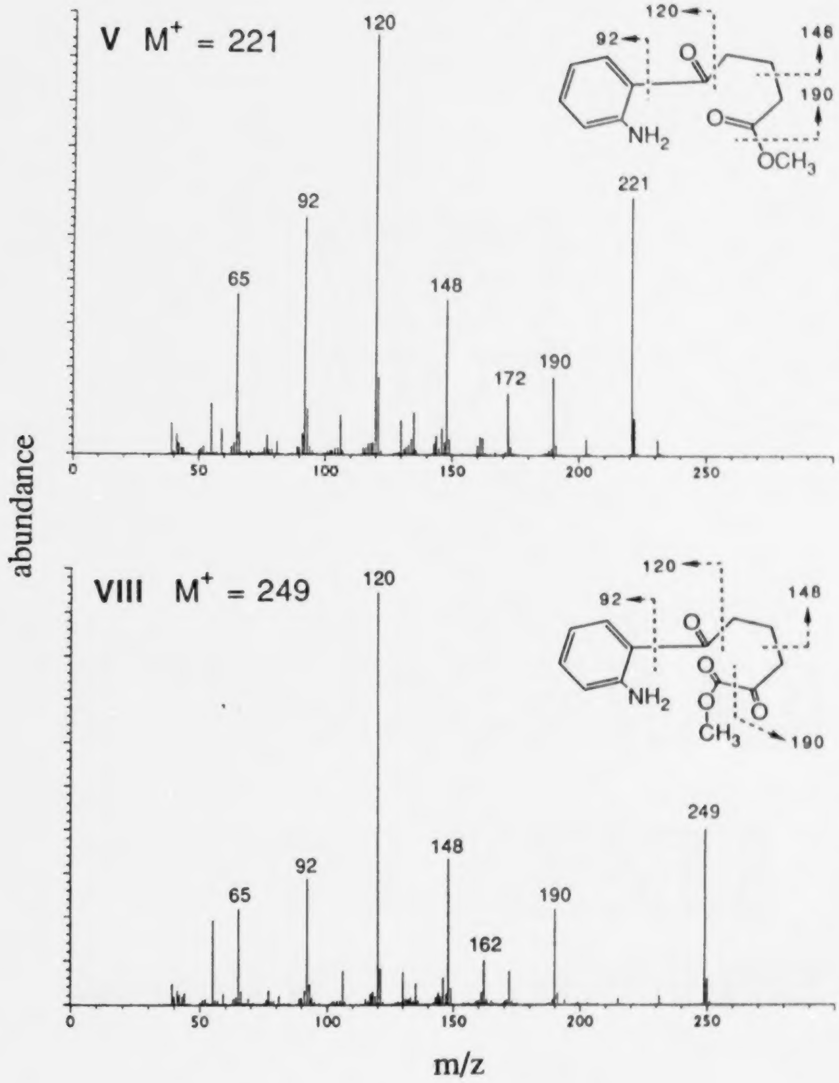


Figure 5.13 The mass spectra and proposed structures of compounds **V** and **VIII**.

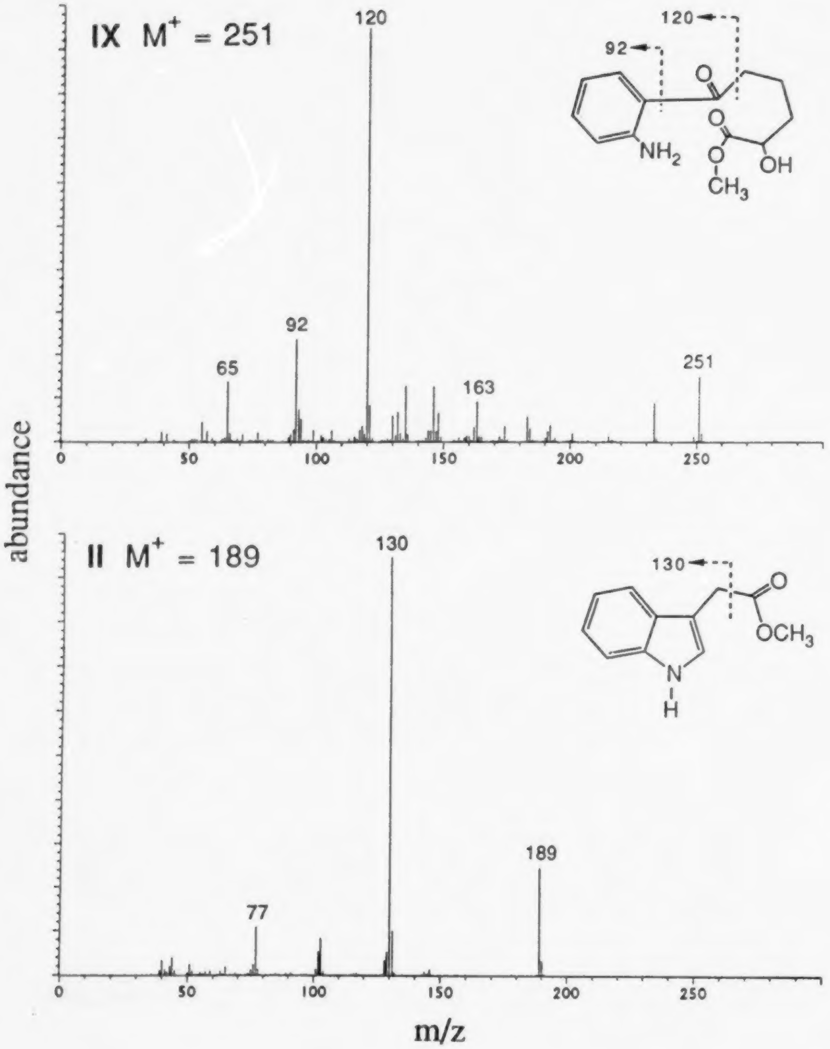


Figure 5.14 The mass spectra and proposed structures of compounds **IX** and **II**.

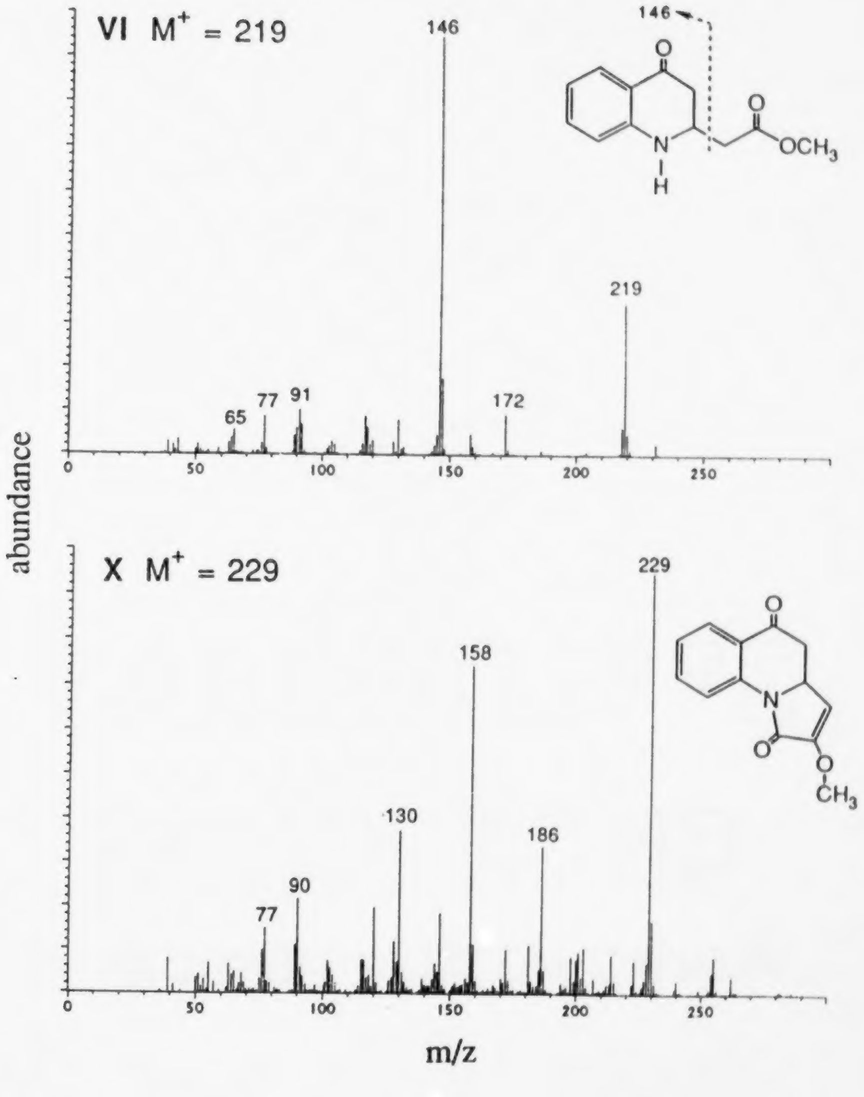


Figure 5.15 The mass spectra and proposed structures of compounds **VI** and **X**.

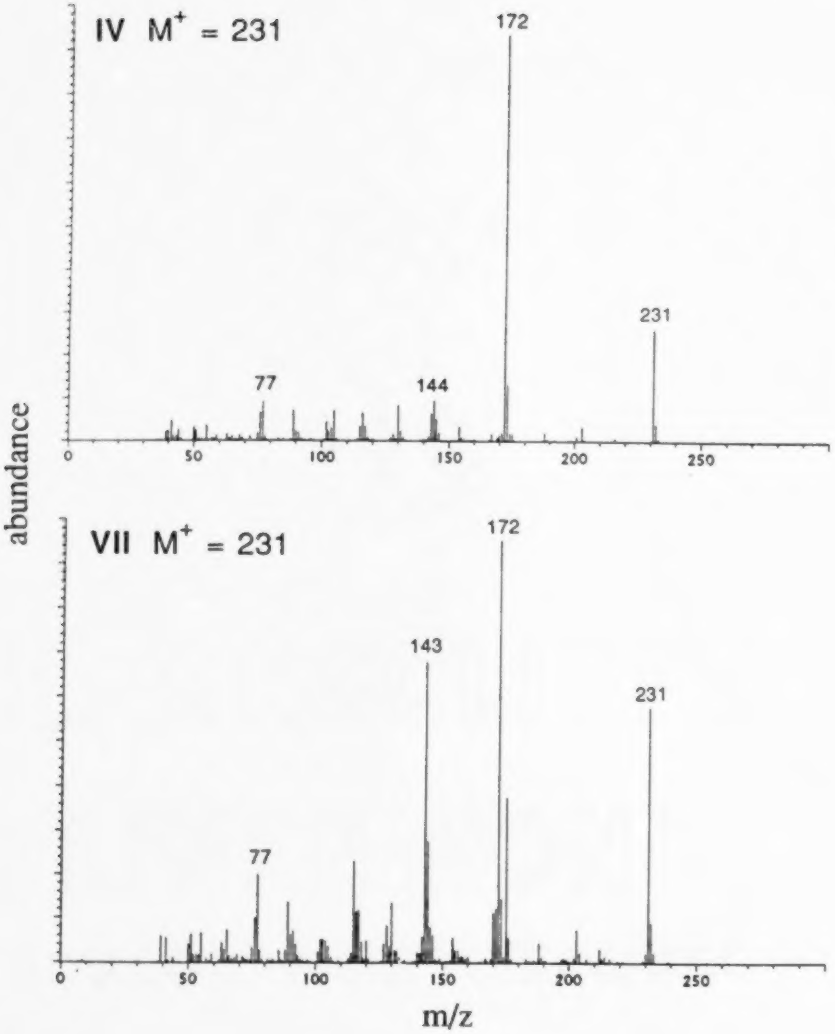


Figure 5.16 The mass spectra of compounds **IV** and **VII**.

and **VII**, for which no structure could be proposed). For each compound, the molecular ion was verified by chemical ionization MS, and the empirical formula was determined by GC-high-resolution MS. Where possible, the presence of functional groups was verified by GC-FTIR. This technique is much less sensitive than GC-MS, therefore good quality FTIR spectra could not be obtained for some metabolites which were not abundant.

5.3.7.2.1 Metabolites with aniline-type structures

Five of the mass spectra shown in Figs. 5.12 to 5.16 have common ions in the low mass range. Three of these [compounds **V**, **VIII** (Fig. 5.13), and **IX** (Fig. 5.14)] have the base peak at m/z 120, and abundant fragment ions at m/z 92 and 65. Compound **III** (Fig. 5.12) also has these fragment ions, although m/z 120 is not the base peak. Compound **I** (Fig. 5.12) has a base peak at m/z 119, and fragment ions at m/z 92 and 65. The fragment at m/z 65, the $C_5H_5^+$ ion, implies an aniline-type structure (Shriner *et al.*, 1980). Indeed, the mass spectrum of the methyl ester of anthranilic acid (M^+= 151), an aniline-type compound, showed the following ions: m/z 120 (30%), 119 (100%), 92 (72%) and 65 (49%). The mass spectrum of an authentic standard of the methyl ester of DL-kynurenine (DL-2-amino-4-(2-aminophenyl)-4-oxobutanoic acid, M^+= 222), another aniline-type compound, also showed ions at m/z 120 (100%), 92 (30%), and 65 (22%). Thus, these five compounds were likely anilines, and were presumed to contain the 2-aminobenzoyl [$(NH_2)C_6H_4(C{=}O)$–] moiety.

The exact mass of compound **I** was 193.07390, corresponding to a formula of $C_{10}H_{11}NO_3$, which suggested that it was the methyl ester of 3-(2-aminophenyl)-3-oxo-propanoic acid. The fragmentation pattern shown in Fig. 5.12 is consistent with this structure. The fragment at m/z 65, the $C_5H_5^+$ ion, implied an aniline-type structure and the ion at m/z 151 would result from the loss of $CH_2{=}C{=}O$, which is a commonly lost fragment (Silverstein *et al.*, 1991). GC-FTIR analysis showed two absorptions at 1709 and 1724 cm^{-1}, indicating the presence of two carbonyl groups.

Compound **III** had an exact mass of 189.07898, corresponding to the formula $C_{11}H_{11}NO_2$. The fragmentation pattern (Fig. 5.12) was consistent with the metabolite being 5-(2-aminophenyl)-5-oxo-pent-3-enal. The ion at m/z 161 $(M-28)^+$ would result from the loss of C=O. The FTIR spectrum showed the presence of two absorptions at 1721 and 1689 cm^{-1}, indicating two carbonyl groups. As an aldehyde, compound **III** should not react with diazomethane and was detected by GC before and after treatment with diazomethane.

Compound **V** had an exact mass of 221.10519, giving a molecular formula of $C_{12}H_{15}NO_3$. These data suggested that it was the methyl ester of 5-(2-aminophenyl)-5-oxo-pentanoic acid, and the predicted fragmentations shown in Fig. 5.13 were consistent with the major ions in the mass spectrum. From GC-FTIR analysis, two absorptions were observed at 3473 and 3400 cm^{-1}, which are characteristic of the asymmetric NH_2 stretching bands of aryl amines (3548 to 3459 cm^{-1}) in the vapor phase (Lin-Vien *et al.*, 1991). The FTIR spectrum also showed two absorptions at 1770 and 1705 cm^{-1}, which indicated the presence of two carbonyl groups. An authentic sample of 5-(2-aminophenyl)-5-oxo-pentanoic acid was synthesized and derivatized with diazomethane to form its methyl ester. The mass spectrum and GC retention time of this standard matched those of compound **V**.

From GC-high-resolution MS, compound **VIII** ($M^+ = 249.10011$) was found to have the molecular formula $C_{13}H_{15}NO_4$, and was presumed to be the methyl ester of 6-(2-aminophenyl)-2,6-dioxo-hexanoic acid. The suggested fragmentation pattern of this molecule (Fig. 5.13) agreed with that observed in the mass spectrum. Absorptions at 3524 and 3360 cm^{-1} in the FTIR spectrum indicated the presence of $-NH_2$, and three strong absorptions (1771, 1742 and 1665 cm^{-1}) indicated the presence of three C=O groups, in agreement with the proposed structure of compound **VIII**.

Compound **IX** was tentatively identified as the methyl ester of 6-(2-aminophenyl)-2-hydroxy-6-oxo-hexanoic acid (Fig. 5.14). Its molecular formula, derived from the exact mass of 251.11575, was $C_{13}H_{17}NO_4$. The low abundance of this compound gave a poor

FTIR spectrum, but it clearly showed two strong absorptions at 1702 and 1665 cm^{-1} corresponding to the stretching of two C=O groups.

5.3.7.2.2 Metabolites with indole-type structures

Compound **II** had an exact mass of 189.07587, corresponding to the empirical formula $C_{11}H_{11}NO_2$. This compound was unequivocally identified as the methyl ester of indole-3-acetic acid (Fig. 5.14) because it had the same GC retention time and mass spectrum as an authentic standard.

This finding led to experiments examining the growth of isolate LD2 on indole-3-acetic acid and related compounds. Indeed, LD2 was able to use indole-3-acetic acid (250 mg/L) as a growth substrate in B-N medium and degrade it nearly to completion within 12 h of incubation (Fig. 5.17). When carbazole and indole-3-acetic acid were both added as substrates for isolate LD2 in B-N medium, both were used simultaneously with indole-3-acetic acid disappearing by 16 h and carbazole by 72 h. These results were similar to those observed with carbazole and anthranilic acid (Fig. 5.9). An experiment in which isolate LD2 was grown with both indole-3-acetic acid and anthranilic acid as substrates in B-N medium (150 mg/L each) showed the simultaneous removal of both compounds by 12 h as determined by HPLC (Fig. 5.18).

Because isolate LD2 was able to use indole-3-acetic acid as a growth substrate, other indole derivatives were tested. Using turbidity (OD_{600}) as a measure of growth, LD2 grew well on *trans*-indole-3-acrylic acid, indole-3-pyruvic acid, indole-3-carboxylic acid, and indole-3-carboxaldehyde and degraded these compounds in B-N medium. Isolate LD2 grew weakly on indole-3-propanoic acid and indole-3-butanoic acid, but biotransformed these two compounds into unidentified products as detected by HPLC (this was not observed in the sterile controls). Isolate LD2 did not grow on or degrade indole, L-tryptophan, or DL-kynurenine when these were added as substrates to B-N medium.

From these results, it seemed likely that other metabolites may be indoles or

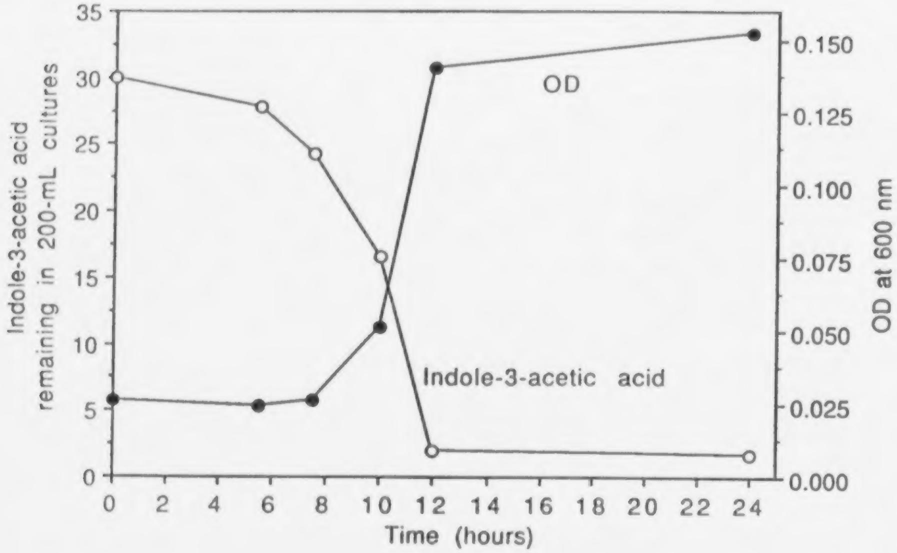


Figure 5.17 Growth of isolate LD2 on indole-3-acetic acid and the loss of this compound in B-N medium.

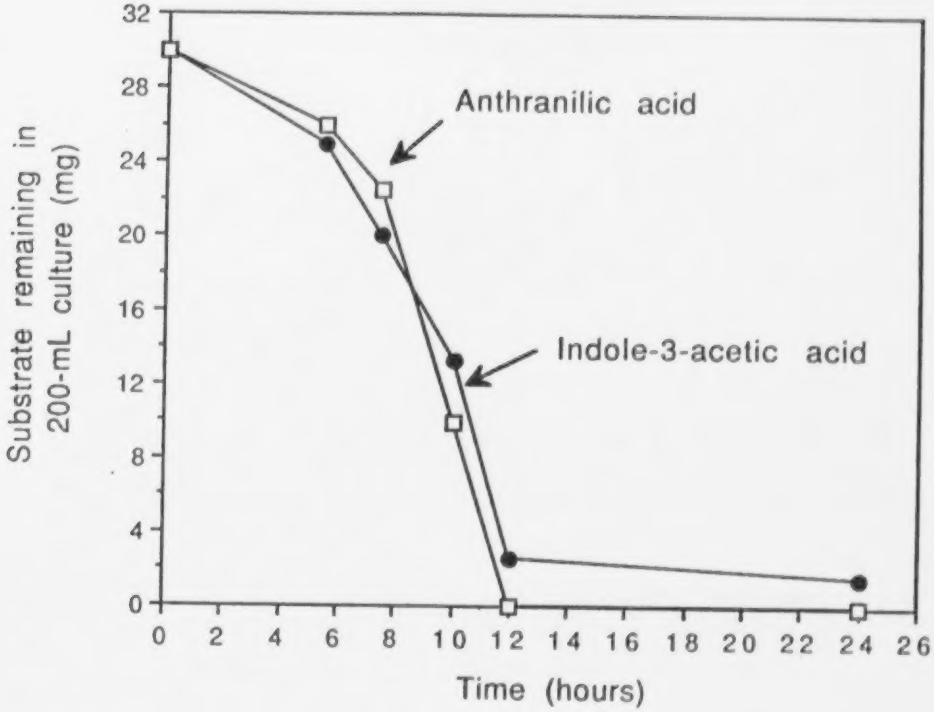


Figure 5.18 Loss of indole-3-acetic acid and anthranilic acid from an LD2 culture when these compounds were added as a mixture in B-N medium.

substituted indoles. Initially, four of the metabolites (Compounds **IV**, **VI**, **VII** and **X**) were postulated to be oxindoles on the basis of their molecular formulae from high-resolution MS, and the presence of two or three carbonyl absorptions in their FTIR spectra. For example, compound **VI** was thought to be the methyl ester of oxindole-3-propanoic acid which would have a molecular mass of 219 (Fig. 5.19a). However, this compound was synthesized and its mass spectrum (Fig. 5.20a) and GC retention time were different from those of compound **VI** (Fig. 5.15). The molecular ion of the methyl ester of oxindole-3-propanoic acid was indeed at m/z 219, although the base peak at m/z 145 and the presence of a major fragment ion at m/z 187 differed from the fragmentation pattern of compound **VI** (Fig. 5.15). Oxindole-3-acetic and oxindole-3-butanoic acids were also synthesized and treated with diazomethane. The base peak of the methyl ester of oxindole-3-acetic acid was also at m/z 145 (Fig. 5.20b), whereas the base peak of the methyl ester of oxindole-3-butanoic acid was at m/z 146 (Fig. 5.20c). Because none of the mass spectra shown in Figs. 5.15 and 5.16 (with the exception of compound **VI**) had base peaks at m/z 145 or 146, none of the metabolites was subsequently considered to be an oxindole.

Although none of the metabolites were considered to be oxindoles, the synthesis of gram quantities of oxindole-3-acetic acid permitted growth and degradation studies with this compound. Isolate LD2 and a sterile control were incubated with oxindole-3-acetic acid (100 mg/L) and samples were removed over time to measure growth by OD_{600} and substrate loss by HPLC. Isolate LD2 did not grow on oxindole-3-acetic acid, but compared to the sterile control, only 6% of the added oxindole-3-acetic acid remained in the LD2 culture after 7 days of incubation. Thus, isolate LD2 was capable of biotransforming oxindole-3-acetic acid, although no biotransformation products were detected by HPLC.

In order to determine whether compounds **IV**, **VI**, **VII**, or **X** were indole-type compounds, three indole derivatives were analyzed by GC-MS. The mass spectra of the methyl esters of indole-3-acetic acid, indole-3-propanoic acid, and indole-3-butanoic acid showed similar fragmentation patterns with a base peak at m/z 130 (Fig. 5.21a, b, and c,

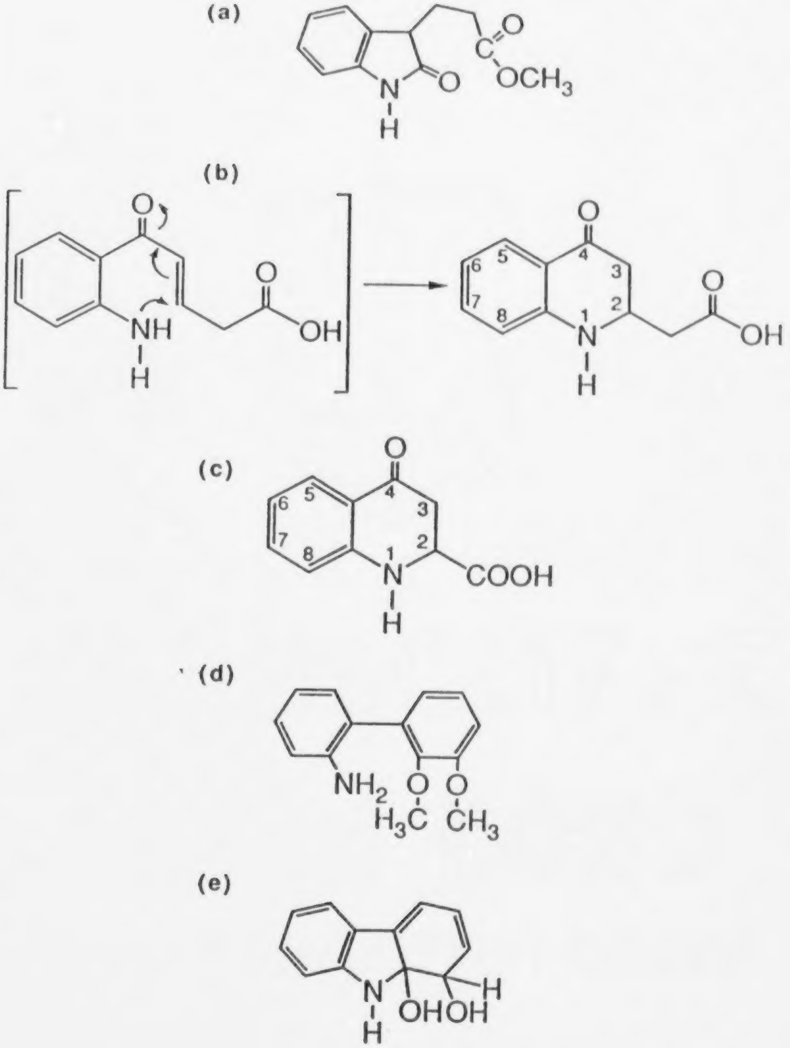

(a)
O
C
OCH$_3$
N
H
O
(b)
O
NH
H
O
OH
O
4
5
6
3
7
2
8
1
N
H
O
OH
(c)
O
4
5
6
3
7
2
8
1
N
H
COOH
(d)
NH$_2$
O
H$_3$C
O
CH$_3$
(e)
N
H
OH
OH
H

Figure 5.19 Structures of some compounds discussed in the text: (a) oxindole-3-propanoic acid; (b) the formation of compound **VI**; (c) kynurenine yellow; (d) dimethyl ether of 2'-aminobiphenyl-2,3-diol; (e) 1,9a-dihydroxy-1-hydrocarbazole.

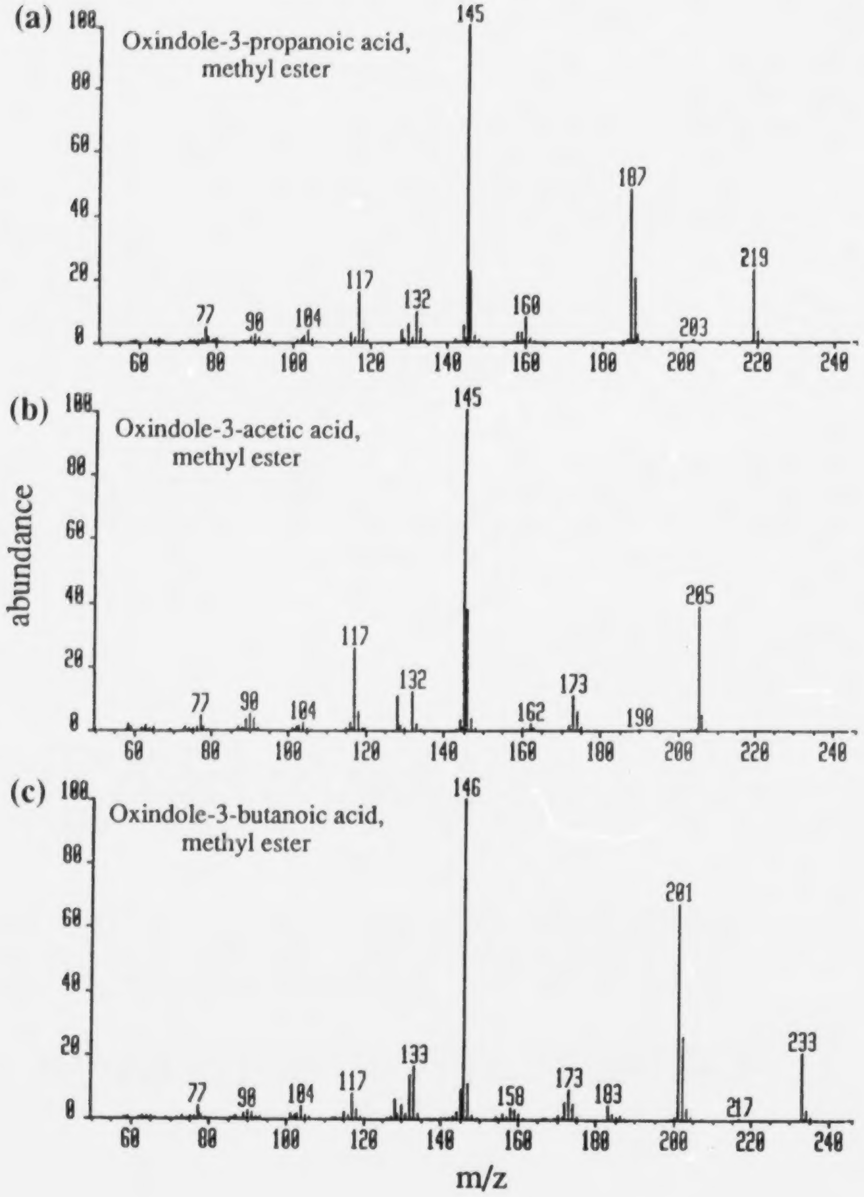


Figure 5.20 The mass spectra of the methyl esters of three synthesized oxindole derivatives showing the base peak at m/z 145 or 146.

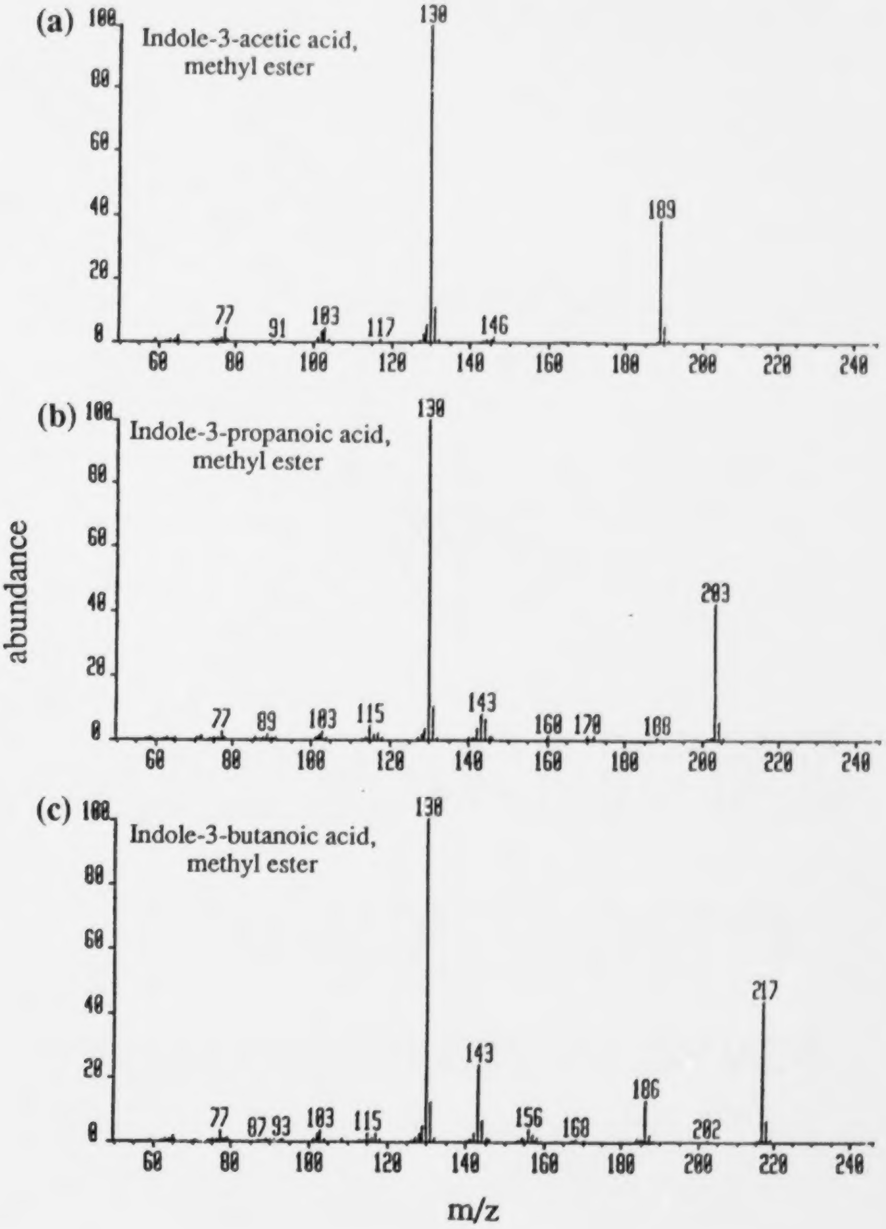


Figure 5.21 The mass spectra of the methyl esters of three indole derivatives showing the base peak at m/z 130.

respectively). This was not observed in the mass spectra of compounds **IV**, **VI**, **VII**, or **X** (Figs. 5.15 and 5.16). Therefore, it was unlikely that these compounds were substituted indoles.

5.3.7.2.3 Metabolites identified by NMR

Fortuitously, when isolate LD2 was grown in the presence of carbazole and dibenzofuran, and the acidified culture supernatant extracts were derivatized with diazomethane, two large nitrogen-containing peaks were observed by GC. These two peaks were found to elute at the same retention times as compounds **VI** and **X** (Fig. 5.11) and had the same mass spectra as previously seen when carbazole was present as the sole substrate (Fig. 5.15). Because these carbazole metabolites were found to accumulate in the medium in the presence of dibenzofuran, it was possible to purify them in sufficient amounts by preparative TLC for NMR analysis. Approximately 50 µg of compound **VI** and 1 mg of compound **X** were purified as their methylated derivatives. The small amount of **VI** did not permit a characterization in the ^{13}C domain but a full proton data set was obtained. Compound **X** was analyzed completely in both domains.

Compound **VI** had an exact mass of 219.08954, corresponding to the formula $C_{12}H_{13}NO_3$. The FTIR spectrum showed a single absorption at 3474 cm^{-1} which is characteristic of the N-H stretch of a secondary amine in vapor phase (Lin-Vien *et al.*, 1991). Once it was confirmed that this metabolite was not the methyl ester of oxindole-3-propanoic acid, a new structure was sought. It had been observed in the bacterial degradation of dibenzofuran that some unstable microbially-produced metabolites could spontaneously cyclize, yielding artifacts which were chemically stable and could be identifed (Fortnagel *et al.*, 1990; Strubel *et al.*, 1991). By analogy to these reactions, compound **VI** was predicted to be the methyl ester of the cyclization product of 5-(2-aminophenyl)-5-oxo-penta-3-enoic acid. Indeed, one- and two-dimensional GCOSY proton NMR data confirmed that compound **VI** was the methyl ester of 2-(2,3-dihydro-4-

oxo-1H-quinoline-2-yl) acetic acid (Fig. 5.15). Chemical shifts and data interpretation are included in Appendix B.

Compound **VI** was presumably formed spontaneously by intramolecular Michael addition of the nucleophilic N atom to the α,β-unsaturated ketone side chain of enzymatically-produced 5-(2-aminophenyl)-5-oxo-penta-3-enoic acid (in brackets, Fig. 5.19b). In addition to having been observed in dibenzofuran biodegradation (Fortnagel *et al.*, 1990; Strubel *et al.*, 1991), this kind of intramolecular, chemical cyclization reaction has been reported to occur in the formation of flavanones from chalcones during secondary metabolism in higher plants (Haslam, 1974). The base peak at m/z 146 (Fig. 5.15), indicative of the 2,3-dihydro-1H-quinolin-4-one moiety of compound **VI**, has been previously seen in the mass spectrum of kynurenine yellow (2-carboxy-2,3-dihydroquinolin-4-one, Fig. 5.19c), a compound similar in structure to compound **VI** (Brown, 1967; Tokuyama *et al.*, 1967).

The mass spectrum of compound **X** (Fig. 5.15) is remarkably similar to a spectrum presented by Ouchiyama *et al.* (1993). Both have a molecular ion of m/z 229 and major fragment ions at m/z 186 and 158. On the basis of low-resolution MS, Ouchiyama *et al.* (1993) tentatively identified this compound as dimethylated 2'-aminobiphenyl-2,3-diol, which has the molecular formula $C_{14}H_{15}NO_2$ (Fig. 4.19d). High-resolution MS of compound **X** gave a mass of 229.07252, which is consistent with the formula $C_{13}H_{11}NO_3$. The FTIR spectrum of this compound showed two absorptions at 1752 and 1704 cm^{-1} indicating the presence of two C=O groups, but no absorptions were seen in the region of 3000 to 4000 cm^{-1} where N-H stretches occur in the vapor phase. In addition, repeated attempts in our laboratory to methylate the model compound 2,3-dihydroxybiphenyl with diazomethane failed, thus providing further evidence that compound **X** was not the dimethylated derivative of 2'-aminobiphenyl-2,3-diol.

Based on the mass and molecular formula determined from GC-high-resolution MS as well as on FTIR data, a number of possible structures for compound **X** were proposed, many of which were indole-type structures with aldehyde groups. However, after analyzing the sample by proton NMR, none of these structures was correct. Several other NMR experiments were conducted in order to elucidate the structure of compound **X**. Data interpretation was challenging, especially because many of the postulated structures could not be explained as having a biological origin. Furthermore, these structures did not completely agree with all of the NMR data obtained. After exhausting most available one- and two-dimensional ^1H and ^{13}C NMR techniques, and much discussion between Dr. A. Otter and myself, compound **X** was unequivocally identified as 3a,4-dihydro-2-methoxy-pyrrolo[1,2-a]quinoline-1,5-dione (Fig. 5.15).

It is proposed that compound **X** is an artifact that was formed chemically from an unstable, enzymatically-produced carbazole degradation product, namely, 6-(2-aminophenyl)-2-hydroxy-6-oxo-hexa-2,4-dienoic acid. This open-ring compound has been tentatively identified as a carbazole metabolite (Ouchiyama *et al.*, 1993). Figure 5.22 shows the postulated formation of compound **X** from this unstable compound in which intramolecular Michael addition occurs to form a six-membered ring in a manner analogous to that proposed for compound **VI** (Fig. 5.19b). The unstable α-hydroxy acid side chain then cyclizes to form a five-membered ring by eliminating H_2O. Upon treatment with diazomethane, the acidic hydroxyl group becomes methylated to form compound **X** (Fig. 5.22). It is proposed that the intramolecular Michael addition to form the six-membered ring occurs spontaneously during incubation at neutral pH and the second cyclization to the five-membered ring results from the elimination of H_2O upon acidification of LD2 culture supernatant prior to extraction.

It is possible that the formation of the six-membered ring is catalyzed by the diazomethane reaction, however, this is not believed to be the case for a few reasons. First, when BF_3-methanol was used to derivatize LD2 acid extracts (methylates -COOH groups), compound **X** was still detected, which suggested that diazomethane did not catalyze the cyclization reaction. Secondly, when acetic anhydride was reacted with LD2

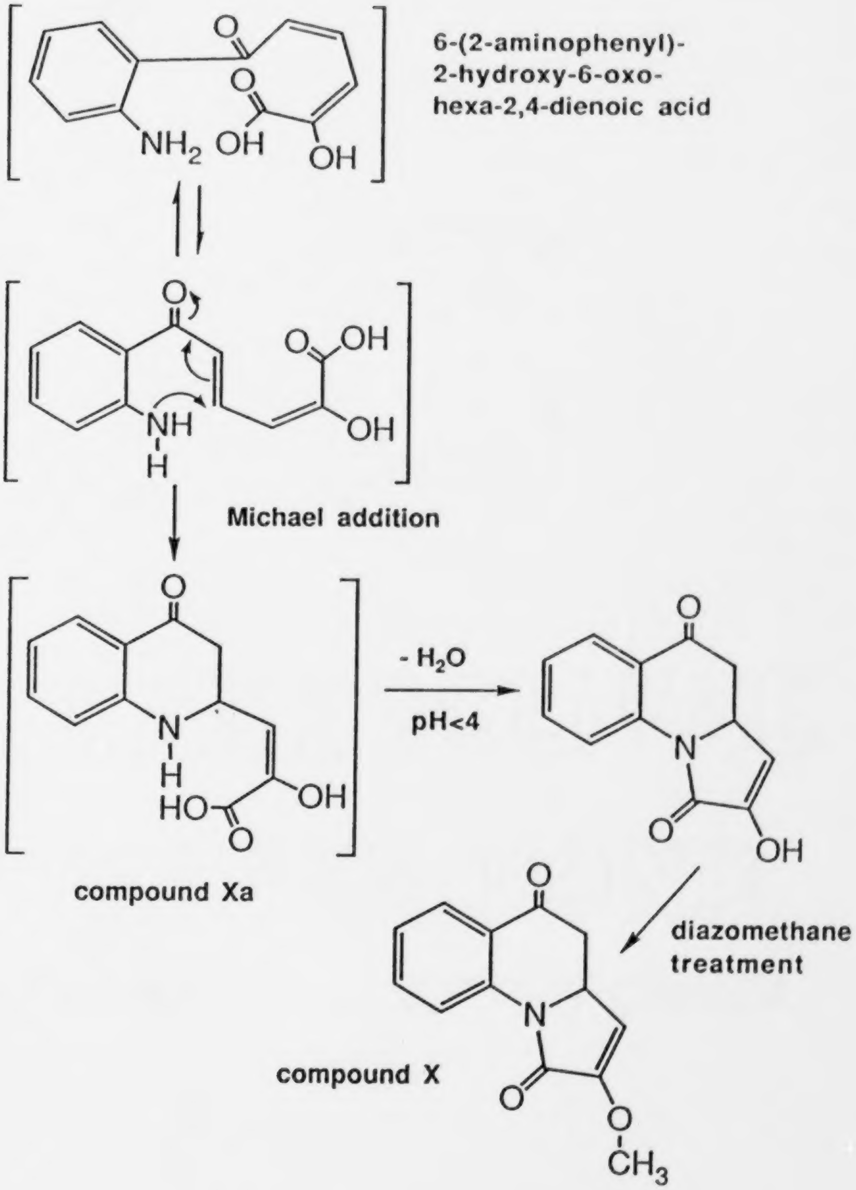

6-(2-aminophenyl)-
2-hydroxy-6-oxo-
hexa-2,4-dienoic acid
NH2
OH
OH
O
O
NH
H
OH
OH
Michael addition
N
H
HO
OH
compound Xa
- H2O
pH<4
N
OH
diazomethane
treatment
compound X
O
CH3

Figure 5.22 The proposed formation of compound **X** from 6-(2-aminophenyl)-2-hydroxy-6-oxo-hexa-2,4-dienoic acid.

acid extracts (acetylates -OH groups) instead of diazomethane, an acetylated derivative of this compound was observed by GC-low-resolution MS ($M^+= 257$). This suggested that formation of the six-membered ring had occurred prior to derivatization.

Furthermore, when underivatized LD2 acid extracts were analyzed by GC using a DB-1 capillary column, a peak eluted from the GC-MS which had the molecular formula ($C_{12}H_9O_3N$) and mass of underivatized compound **X** ($M^+= 215.05823$). The mass spectrum had a similar fragmentation pattern as that of the diazomethane-treated compound. For example, the major ions for compound **X** were at m/z 229, 186, and 158, whereas the major ions for underivatized compound **X** were at m/z 215, 187, and 159.

In an attempt to detect 6-(2-aminophenyl)-2-hydroxy-6-oxo-hexa-2,4-dienoic acid or the initial six-membered ring cyclization product [compound **Xa**, 3-(2,3-dihydro-4-oxo-1H-quinoline-2-yl)-2-hydroxyprop-2-enoic acid, Fig. 5.22], LD2 cultures incubated with carbazole were extracted at pH values from 2 to 7, derivatized with diazomethane, and analyzed by GC-MS. Some diazomethane-treated samples which were extracted at pH 2 showed a nitrogen-containing peak which eluted shortly after that of compound **X**. Upon analyzing by GC-high-resolution MS, this peak was found to have an exact mass of 261.09647 and a molecular formula of $C_{14}H_{15}O_4N$. The major fragment was at m/z 146, similar to that seen for the mass spectrum of compound **VI** (Fig. 5.15). On the basis of this data, a structure corresponding to the dimethylated derivative of 3-(2,3-dihydro-4-oxo-1H-quinoline-2-yl)-2-hydroxyprop-2-enoic acid was proposed, although this compound was not purifed for analysis by NMR. The mass spectrum and postulated structure are shown in Fig. 5.23. This structure is analogous to "DBF-5" (Fig. 5.7) which was tentatively identified as a metabolite produced when LD2 was incubated with carbazole and dibenzofuran.

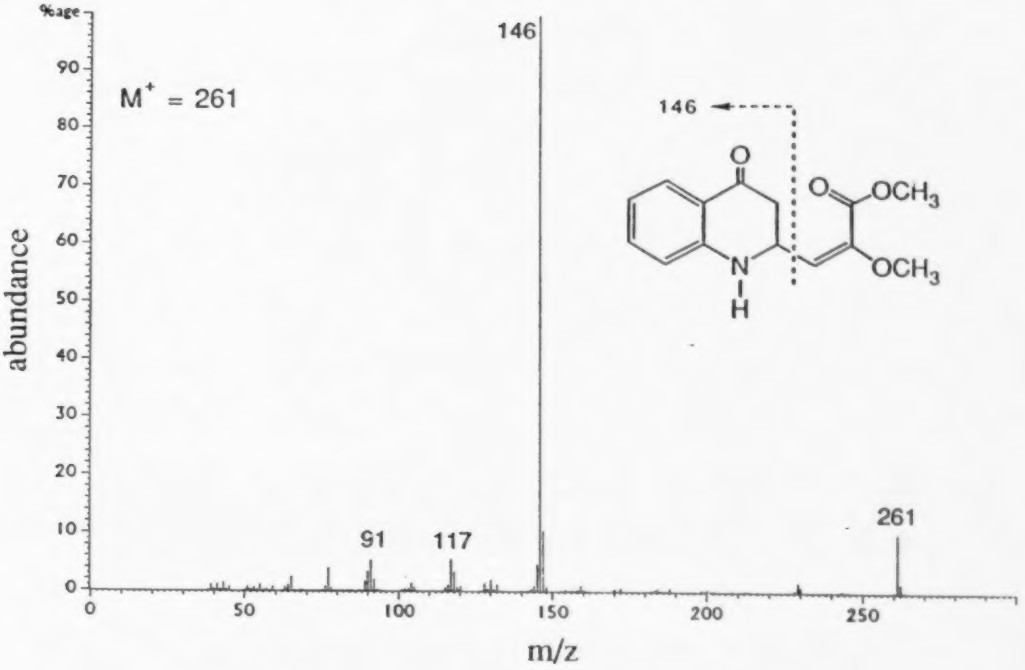


Figure 5.23 The mass spectrum and proposed structure of dimethylated compound **Xa** originating from 6-(2-aminophenyl)-2-hydroxy-6-oxo-hexa-2,4-dienoic acid.

5.3.7.2.4 Other metabolites

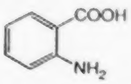
Compounds **IV** and **VII** have similar mass spectra (Fig. 5.16). Both compound **IV**, having an exact mass of 231.08954 and compound **VII**, having an exact mass of 231.08802, correspond to the formula $C_{13}H_{13}NO_3$. The FTIR spectrum of compound **IV** showed two absorptions at 1758 and 1666 cm^{-1}, indicating the presence of two carbonyl groups, as well as two absorptions in the region 3300 to 3500 cm^{-1} which suggested the presence of an $-NH_2$ group (no FTIR spectrum was available for compound **VII**). Although the FTIR data suggests an aniline-type structure, a base peak of m/z 120 was not observed in the mass spectrum of compound **IV** as would be expected for this kind of compound. The base ion in the mass spectra of both of these compounds is m/z 172, which corresponded to the loss of $O{=}C{-}OCH_3$. It is possible that these compounds are 2-aminobenzoyl-type compounds which have cyclized in a manner analogous to compounds **VI** and **X**, but this conflicts with the FTIR observations. Because of conflicting data, no reasonable structures could be assigned for these compounds.

Another possible carbazole metabolite was observed when underviatized LD2 acid extracts were analyzed by GC using a DB-1 capillary column. A large peak eluting later than underivatized compound **X** was observed, and high resolution GC-MS showed it to have a mass of 199.06417 and a molecular formula of $C_{12}H_9NO_2$. This formula corresponds to what might be a dihydroxycarbazole compound.

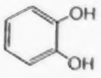
For easy reference, the designations, structures, and names of all the carbazole metabolites tentatively or positively identified in this work are summarized in Fig. 5.24.

5.3.8 Metabolites detected from other carbazole-degrading cultures

After several carbazole metabolites had been tentatively or positively identified in LD2 supernatant extracts, the acidified supernatants of mixed carbazole-degrading populations were examined for the presence of these and other metabolites, based on matching GC retention times with a nitrogen-selective detector. The mixed cultures CM,

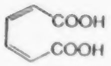


anthranilic acid



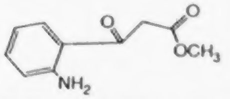
catechol

Q



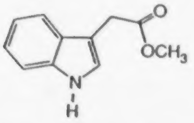
cis, cis-muconic acid

I



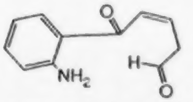
3-(2-aminophenyl)-3-oxo-
propanoic acid,
methyl ester

II



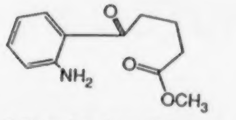
indole-3-acetic acid,
methyl ester

III



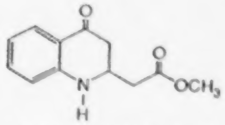
5-(2-aminophenyl)-5-
oxo-pent-3-enal

V



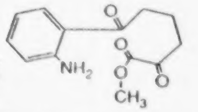
5-(2-aminophenyl)-5-oxo-
pentanoic acid,
methyl ester

VI



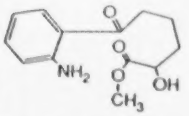
2-(2,3-dihydro-4-oxo-1H-quinoline-2-yl)
acetic acid,
methyl ester

VIII



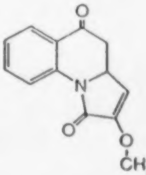
6-(2-aminophenyl)-2,6-dioxo-
hexanoic acid,
methyl ester

IX

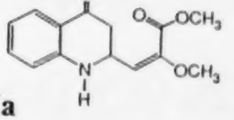


6-(2-aminophenyl)-2-hydroxy-
6-oxo -hexanoic acid,
methyl ester

X



3a,4-dihydro-2-methoxy-
pyrrolo[1,2-a]quinoline-1,5-dione



Xa

3-(2,3-dihydro-4-oxo-1H-quinoline-2-yl)-
2-hydroxyprop-2-enoic acid,
dimethylated

Figure 5.24 Summary of the metabolites identified from carbazole degradation by isolate LD2. Anthranilic acid, catechol, and *cis,cis*-muconic acid were identified as BSA derivatives but are shown here in their underivatized forms.

DC, D5, CB1 Mix, and CB1 Mix as well as CB1 Pure, were found to produce many of the same carbazole metabolites as isolate LD2, and Table 5.4 indicates which products were detected in the extracts of these cultures (denoted by checkmarks). Compound **VI** was the most commonly detected metabolite, and compound **X** was only evident in the D5 culture. Indole-3-acetic acid (compound **II**) was present in the supernatant extracts of CB2 Mix and was the only carbazole metabolite detected in the CB1 Pure culture. While only small amounts of many of these compounds were detected in the cultures, compound **V** was a major product of carbazole degradation in the CB1 Mix culture. In addition, a new nitrogen-containing compound, eluting between compounds **VIII** and **IX** (Fig. 5.11) was evident in CB1 Mix, CB2 Mix, and CM culture extracts. No identification of this compound was made and no other nitrogen-containing metabolites were observed.

5.3.9 Metabolites detected from LD2 mutants

Washed cells of isolate LD2 and nine LD2 transposon mutants were incubated with carbazole for 24 h. Acidified supernatants extracts were treated with diazomethane and analyzed by GC for the presence of the metabolites previously identified for isolate LD2. It was hoped that these mutants would be blocked at different enzymatic steps in the carbazole metabolic pathway thus resulting in the accumulation of carbazole degradation products which would allow the differentiation between true carbazole intermediates and products of side reactions. Several of the mutants were able to clear carbazole plates at rates similar to the wild type, and it was surprising to find that the mutants produced several of the same metabolites as wild-type LD2 (Table 5.4). The accumulation of one specific metabolite from a particular mutant was not observed.

Only compounds **I**, **II**, **IV**, **VII**, and **IX** were present in wild-type LD2 culture extracts when TSB-grown washed cells were incubated with carbazole (Table 5.4). The most commonly seen compounds were **I**, **IV**, and **IX**, which accumulated in the mutants but not in the wild-type. The mutants PMB 11, 12, 13, and 17 produced similar

Table 5.4 Metabolites identified in other carbazole-degrading cultures and LD2 mutants.

CULTURE	I	II	III	IV	V	VI	VII	VIII	IX	X
GROWING CULTURES										
DC	√		√	√		√	√	√		
CM						√				
D5	√			√	√	√		√		√
CB1 Mix					√	√				
CB1 Pure		√								
CB2 Mix		√				√				
WASHED CELLS										
LD2	√	√		√			√		√	
PMB 11	√		√	√			√		√	
PMB 12	√			√			√		√	
PMB 13	√			√			√		√	
PMB 17	√			√			√		√	
PMB 18	√	√		√	√	√	√	√	√	
PMB 19	√	√		√		√	√	√	√	
PMB 20	√			√			√	√	√	
PMB 21	√	√		√	√			√	√	
PMB 22	√			√			√	√	√	

metabolites as wild-type LD2, and compounds **II**, **V**, **VI**, **VIII**, and **X** were not seen in these mutants extracts. Compound **II** was present in extracts of PMB 18, 19, and 21, compound **V** was evident only in PMB 18 and 21, compound **VI** appeared only from PMB 18 and 19, and compound **VIII** accumulated only in mutants PMB 18 to 22. From this experiment, it was difficult to determine whether the mutants were blocked in any particular step of carbazole degradation because not all of the expected carbazole metabolites were observed in the extracts of wild-type LD2.

5.4 DISCUSSION

Chen *et al*. (1993) demonstrated that some free-living N_2-fixing bacteria could grow on a variety of aromatic compounds while fixing atmospheric N_2. The carbazole-degrading bacterium LD2 isolated in this study showed no nitrogenase activity, and therefore used carbazole as its sole source of carbon, nitrogen, and energy. The isolate was identified as a *Pseudomonas* sp. Other bacteria that grow on carbazole include several *Pseudomonas* species (Ouchiyama *et al*., 1993; Resnick and Gibson, 1993; Hisatsuka and Sato, 1994; Aislabie, 1994a) a *Xanthomanas* sp. (Grosser *et al*., 1991), and a *Comamonas* sp. (Aislabie, 1994b).

Isolate LD2 reached maximum growth on carbazole within 24 h of incubation while degrading 65% of the carbazole added (250 mg/L) to nitrogen-free mineral medium. Within 48 h, 85% was degraded, and degradation was complete within 96 h. The *Pseudomonas* spp. CA06 and CA10 isolated by Ouchiyama *et al*. (1993) showed poor growth on carbazole without the addition of dimethyl sulfoxide (DMSO) to solubilize carbazole. When a 5.7 mM solution of carbazole in DMSO was added to the medium, they found complete degradation of carbazole within 18 h by both strains. Our experiments did not use a solvent to disperse carbazole, so the rate of carbazole degradation by isolate LD2 was likely limited by its dissolution rate. Yeast extract was added to the growth medium used by Ouchiyama *et al*. (1993) and was absolutely required for the growth of the two carbazole-degrading isolates of Aislabie (1994b). Yeast extract was not required for the growth of isolate LD2.

The mineralization of carbazole by isolate LD2 was comparable to that of the carbazole-degrading *Xanthomonas* sp. isolated by Grosser *et al*. (1991). Whereas the *Xanthomonas* sp. mineralized ca. 60% of the added ^{14}C-carbazole after 2 days, isolate LD2 mineralized between 40 to 50% of ^{14}C-carbazole within this time. Isolate LD2 was found to degrade carbazole equally well whether or not an inorganic nitrogen source was present in the growth medium as was observed with other carbazole-degrading bacteria described by Finnerty *et al*. (1983).

Isolate LD2 grew on only a few of the aromatic and heterocyclic compounds tested (2 of the 20 compounds listed in Table 5.2). It did not grow on or mineralize benzene, naphthalene, phenanthrene, or anthracene. This appears to be typical of other carbazole-degrading bacteria. For example, none of four isolates described by Finnerty *et al*. (1983) grew on naphthalene; the *Xanthomonas* sp. isolated by Grosser *et al*. (1991) did not mineralize anthracene; and the two *Pseudomonas* spp. described by Ouchiyama *et al*. (1993) could not grow on naphthalene. Isolate LD2 was not able to grow on the carbazole analogs dibenzothiophene, dibenzofuran, or fluorene which is consistent with other reports (Finnerty *et al*., 1983; Ouchiyama *et al*., 1993; Resnick and Gibson, 1993). Strain LD2 utilized some aromatic compounds including anthranilic acid, catechol, 4-hydroxybenzoic acid, and 2,3-dihydroxybiphenyl and simple substrates like glucose, acetate, pyruvate, and succinate. These have been reported as substrates for other carbazole-degrading bacteria (Finnerty *et al*., 1983; Ouchiyama *et al*., 1993; Resnick and Gibson, 1993). It was interesting that when isolate LD2 was supplied with carbazole as a nitrogen source and glucose or acetate as a carbon source, it preferred to utilize carbazole as both a carbon and nitrogen source, even when inorganic nitrogen sources were present in the growth medium.

The bacterial oxidation of dibenzothiophene has been shown to occur at carbons 1 and 2 (see Fig. 5.4, note different numbering conventions for carbazole and dibenzothiophene) yielding 1,2-dihydroxydibenzothiophene (Laborde and Gibson, 1977), *cis*-4-[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid and a series of colored products (Kodama *et al.*, 1973). Similarly, the initial bacterial oxidation of many aromatic hydrocarbons is via a 1,2-dioxygenase giving *cis*-1,2-dihydrodiols which leads to the formation of the corresponding catechol intermediates (Gibson and Subramanian, 1984). Recently, Resnick *et al.* (1993) studied the oxidation of carbazole using washed cell suspensions of *Pseudomonas* sp. NCIB 9816/11, that contained naphthalene 1,2-dioxygenase, and *Beijerinckia* sp. B8/36, that contained biphenyl dioxygenase. These cell suspensions yielded 3-hydroxycarbazole which was presumed to result from the dehydration of an unstable *cis*-carbazole-3,4-dihydrodiol.

Although strain LD2 oxidized dibenzothiophene to a sulfoxide, its inability to produce any colored oxidation products from dibenzothiophene, as observed by others (Kodama *et al.*, 1973; Laborde and Gibson, 1977; Foght and Westlake, 1988), suggested that the initial oxidation of carbazole was different than that reported for the sulfur analog. In their proposed pathway of carbazole degradation by two *Pseudomonas* species, Ouchiyama *et al.* (1993) tentatively identified an early oxidation product of carbazole degradation to be 2'-aminobiphenyl-2,3-diol (shown as the dimethyl ether, Fig. 5.19d). This compound was proposed to result from dioxygenase attack at the 1 and 9a positions, resulting in the formation of 1, 9a-dihydroxy-1-hydrocarbazole (Fig. 5.19e), which was not detected. Spontaneous cleavage between the nitrogen atom and the adjacent hydroxylated carbon atom would restore aromaticity, giving 2'-aminobiphenyl-2,3-diol. This proposed mechanism is analogous to initial steps in dibenzofuran and fluorene degradation (Strubel *et al.*, 1991; Monna *et al.*, 1993; Grifoll *et al.*, 1994) involving initial angular dioxygenation (Engesser *et al.*, 1989). Resnick and Gibson (1993) reported the metabolite 1-hydroxycarbazole from a mutant *Pseudomonas stutzeri* strain, which was thought to have resulted from the rearomatization of the unstable 1,9a-dihydroxy-1-hydrocarbazole. The observation that strain LD2 was able to oxidize dibenzofuran (in the presence of carbazole) to metabolites which would result from such an angular cleavage suggested that this bacterium degraded carbazole in a similar manner.

In their pathway for carbazole degradation, Ouchiyama *et al.* (1993) proposed *meta* cleavage of 2'-aminobiphenyl-2,3-diol to form 6-(2-aminophenyl)-2-hydroxy-6-oxo-hexa-2,4-dienoic acid as a metabolite (Fig. 5.22). It is proposed in this work that compound **X** is formed chemically from this unstable compound under neutral culture conditions via intramolecular Michael addition to form compound **Xa** (Fig. 5.22) in a manner analogous to that proposed for compound **VI** (Fig. 5.19b). It was then postulated that, upon acidification, the unstable α-hydroxy acid side chain cyclizes to a five-membered ring by eliminating H_2O, and subsequent treatment with diazomethane forms compound **X** (Fig. 5.22). The detection of a compound which could be the dimethylated derivative of compound **Xa** (Fig. 5.23) in some LD2 acidified extracts suggests that the formation of the compound **X** ring may not be the ultimate fate of 6-(2-aminophenyl)-2-hydroxy-6-oxo-hexa-2,4-dienoic acid due to sample work-up under acidic conditions. Compound **Xa** was observed when LD2 was incubated with carbazole but not when incubated with carbazole and dibenzofuran. Compound **X** was present in relatively low concentrations in cultures containing only carbazole, but accumulated in the presence of dibenzofuran. Thus, the formation of the five-membered ring under acidic conditions may be due to chemical reactions which are more favorable in the presence of dibenzofuran and in cultures containing only carbazole, this cyclization does not occur as readily. This is just speculation, however, because the structure of the proposed dimethyl ether of compound **Xa** has not been unequivocally identified. Derivatives of 6-(2-aminophenyl)-2-hydroxy-6-oxo-hexa-2,4-dienoic acid were not detected in LD2 culture extracts using any derivatization method or at any pH examined.

Even though the mass spectrum obtained for compound **X** was virtually identical to that of the dimethyl ether of 2'-aminobiphenyl-2,3-diol reported by Ouchiyama *et al.* (1993), GC-high resolution-MS and NMR unequivocally identified this compound to be 3a,4-dihydro-2-methoxy-pyrrolo[1,2-a]quinoline-1,5-dione. It is possible that compound **X** has the same mass spectrum as the dimethyl ether of 2'-aminobiphenyl-2,3-diol, although confirmation of this awaits the synthesis of an authentic standard of the diol.

Compound **X** is likely an artifact resulting from the unstable intramolecular cyclization of 6-(2-aminophenyl)-2-hydroxy-6-oxo-hexa-2,4-dienoic acid suggesting that carbazole is indeed being initially attacked by *Pseudomonas* sp. LD2 via angular dioxygenation as proposed by Ouchiyama *et al.* (1993). The identification of compound **VI** supports this hypothesis, as does the presence of 2-aminobenzoyl-type compounds (**I**, **III**, **V**, **VIII**, and **IX**) detected in the acidified supernatants of isolate LD2 (Fig. 5.24). The presence of metabolites with various lengths of carboxylic acid side chains [C_6 (**VIII**, **IX**, **X**), C_5 (**III**, **V**, **VI**), C_3 (**I**), and C_1 (anthranilic acid)] in culture supernatants shows that loss of carbon occurred during carbazole degradation by isolate LD2. Compound **V**, positively identified as 5-(2-aminophenyl)-5-oxo-pentanoic acid (Fig. 5.24), was reported as a major metabolite in cultures of *Pseudomonas ovalis* UB-1701 grown with carbazole and *n*-alkanes (Yamada *et al.*, 1974a). This compound was given the common name ovalic acid, and acted as a plant hormone to stimulate the growth of rice roots. Compound **VI**, unequivocally identified as 2-(2,3-dihydro-4-oxo-1H-quinoline-2-yl)acetic acid methyl ester (Fig. 5.24), was presumed to cyclize from 5-(2-aminophenyl)-5-oxo-penta-3-enoic acid in a manner analogous to that observed previously for dibenzofuran degradation (Fortnagel *et al.*, 1990; Strubel *et al.*, 1991). Ouchiyama *et al.* (1993) tentatively identified the unstable, uncyclized form of compound **VI** as a metabolite of carbazole degradation (in brackets, Fig. 5.19b). The tentative identification of compound **IX** was also reported (Ouchiyama *et al.*, 1993).

Whether these metabolites are part of the main catabolic pathway for carbazole metabolism in isolate LD2 or whether they are the result of side reactions remains unknown. The mechanisms by which these compounds are produced in LD2 cultures are also unknown (except for the formation of compounds **VI** and **X**), although several are thought to have resulted from enzymatic processes. Based on the identification of these 2-aminobenzoyl-type compounds in LD2 supernatant extracts, Pathway 1 of carbazole degradation by this isolate is proposed in Fig. 5.25. Although compounds **III**, **V**, and **I** are shown to be direct intermediates in the metabolism of carbazole leading to anthranilic acid, it is not known whether this sequence of events actually occurs.

Preliminary experiments in which isolate LD2 was incubated for periods of up to a month showed that some of the 2-aminobenzoyl-type compounds identified in this study did not decrease in concentration over time as would be expected if they were transient carbazole intermediates (data not shown). In addition, compound **VI** persisted in LD2 cultures and several of the other carbazole-degrading cultures tested (Table 5.4). This 2-ringed structure is quite different from that of carbazole and presumably the different bacterial populations would have difficulty metabolizing it. None of the 2-aminobenzoyl-type metabolites identified (except for anthranilic acid) was available in sufficient quantity to test as a growth substrate for isolate LD2. Experiments with LD2 mutants did not aid in identifying which metabolites were actual intermediates in the carbazole degradation pathway.

Isolate LD2 grew well on indole-3-acetic acid and several indole derivatives including *trans*-indole-3-acrylic acid, indole-3-pyruvic acid, indole-3-carboxaldehyde, and indole-3-carboxylic acid. Thus, indole-3-acetic acid, a common plant hormone synthesized by many plants and soil microorganisms (Lebuhn and Hartmann, 1993), may be a key intermediate in the catabolism of carbazole. L-Tryptophan has been found to accumulate in cells incubated with carbazole (Yamada *et al.*, 1974b) and is a precursor in indole-3-acetic acid biosynthesis in rhizobacteria (Lebuhn and Hartmann, 1993). Although

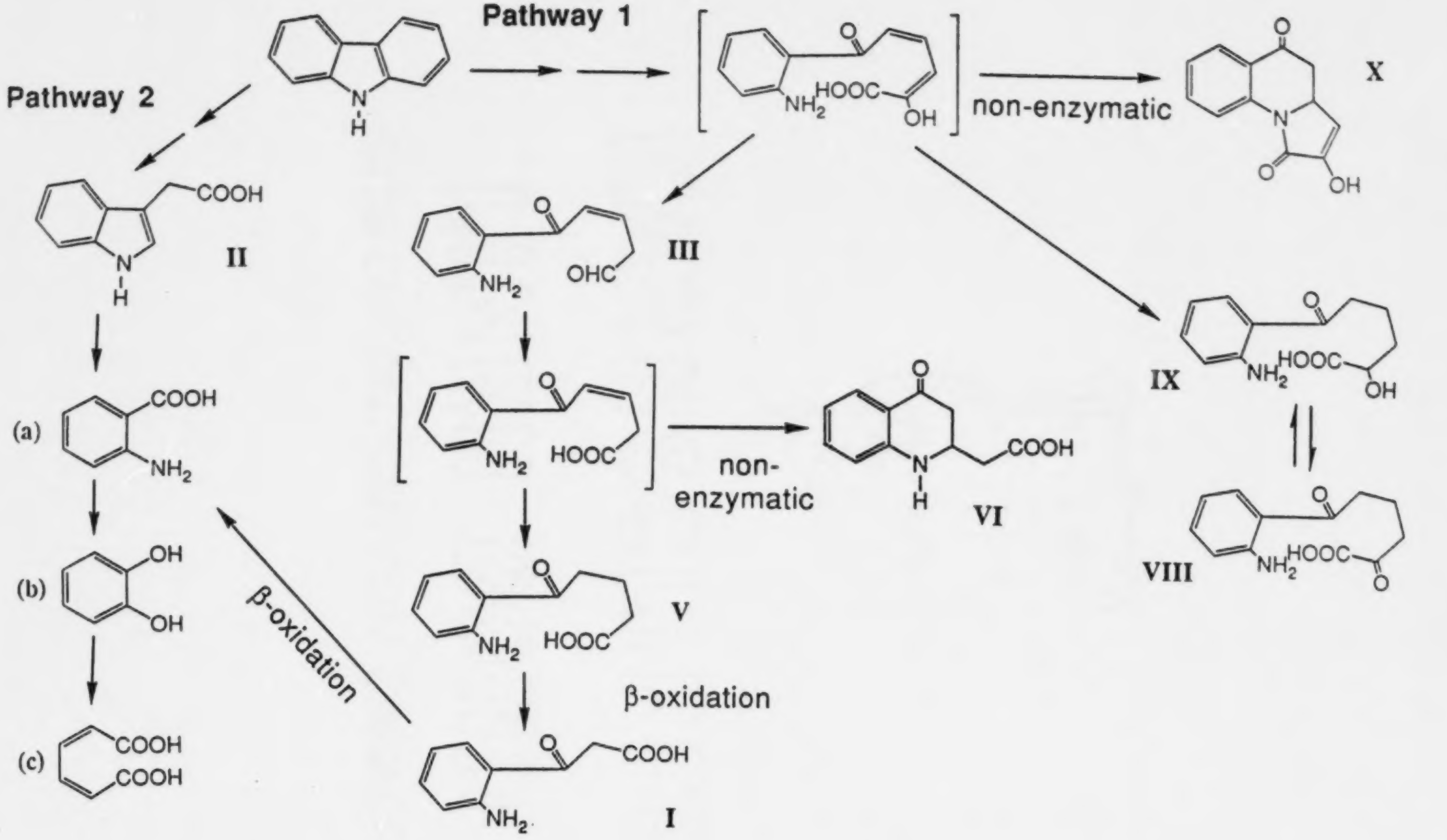

Pathway 1
Pathway 2
non-enzymatic
X
II
III
IX
(a)
non-
enzymatic
VI
VIII
(b)
β-oxidation
V
β-oxidation
(c)
I

Figure 5.25 Proposed pathways of carbazole degradation by isolate LD2 via 6-(2-aminophenyl)-2-hydroxy-6-oxo-hexa-2,4-dienoic acid (Pathway 1; compounds **III**, **V**, **I**) or indole-3-acetic acid (Pathway 2; compound **II**) to anthranilic acid (a), catechol (b), and *cis*, *cis*-muconic acid (c). Compounds **VI**, **VIII**, **IX**, and **X** are thought to originate from 6-(2-aminophenyl)-2-hydroxy-6-oxo-hexa-2,4-dienoic acid.

L-tryptophan did not serve as a sole carbon source or sole carbon and nitrogen source for the growth of isolate LD2, it may still be an intermediate in carbazole degradation leading to the formation of indole-3-acetic acid. Furthermore, anthranilic acid is an intermediate in the degradation of indole-3-acetic acid (Tsubokura *et al.*, 1961; Claus and Kutzner, 1983; Egebo *et al.*, 1991), so its formation in LD2 cultures may have resulted from the metabolism of indole-3-acetic acid and not through the sequential removal of side chain carbon atoms from 6-(2-aminophenyl)-2-hydroxy-6-oxo-hexa-2,4-dienoic acid (Fig. 5.25, to compound **I**). Fig. 5.25 (Pathway 2) shows that carbazole may be metabolized to anthranilic acid via indole-3-acetic acid by isolate LD2.

Recently, Grosser *et al.* (1994) tentatively identified three indole derivatives as metabolites produced by a *Xanthamonas* sp. grown on carbazole as a carbon source. These were reported to be indoleacetic acid, indolepropionaldehyde, and *cis*-indoleacrylic acid. The formation of indole-3-acetic acid by isolate LD2 could also involve a dihydrodiol pathway. Indeed, a compound which may be a dihydroxycarbazole was present in acidified, underivatized LD2 extracts.

The positive identification of anthranilic acid and *cis, cis*-muconic acid in the supernatant extracts of isolate LD2 grown on carbazole confirms the lower pathway proposed for carbazole degradation (Ouchiyama *et al.*, 1993). Anthranilic acid serves as a growth substrate for isolate LD2 and other carbazole-degrading bacteria (Ouchiyama *et al.*, 1993; Resnick and Gibson, 1993; Hisatsuka and Sato, 1994). Ziegler *et al.* (1989) presented three pathways for the metabolism of anthranilic acid, and one of these yields catechol. Although catechol was not detected in the supernatants of carbazole-grown LD2, it was detected as a metabolite in the degradation of anthranilic acid by this bacterium. Furthermore, isolate LD2 used catechol as a growth substrate. *Ortho* cleavage of catechol yields *cis, cis*-muconic acid (Gibson and Subramanian, 1984), which was found in extracts of strain LD2 incubated with carbazole, anthranilic acid, or catechol. *cis, cis*-Muconic acid was reported to be a metabolite of carbazole degradation by Ouchiyama *et al.* (1993), although no evidence for the basis of its identification was presented.

The results of this study of carbazole degradation by *Pseudomonas* strain LD2 support the hypothesis that carbazole is being initially oxidized by angular dioxygenation as has been shown for the analogous compounds fluorene and dibenzofuran. Carbazole metabolites which are similar in structure to those reported by Ouchiyama *et al.* (1993) were detected and tentatively identified. Although 2'-aminobiphenyl-2,3-diol was not detected as an early oxidation product, the unequivocal identification of the methyl ether of its cyclized *meta* cleavage product, 6-(2-aminophenyl)-2-hydroxy-6-oxo-hexa-2,4-dienoic acid, was made. Despite these results, the findings with indole-3-acetic acid and other indole derivatives suggest that an alternative mechanism may be occurring during the catabolism of carbazole by isolate LD2 and this unknown pathway may be the major route for carbon flow (Fig. 5.25). With the exception of indole-3-acetic acid, the metabolites identified in this study may accumulate or serve as substrates for other microorganisms in contaminated environments containing carbazole.

Although extensive growth studies were not done with the mixed carbazole-degrading cultures maintained during this project, several identified metabolites of carbazole degradation by isolate LD2 were observed in supernatant extracts of these mixed populations (Table 5.4). This suggested that carbazole degradation by isolate LD2 was not unusual or unique and that carbazole degradation likely proceeds via similar mechanisms in carbazole-degrading bacteria isolated from contaminated environments in different parts of the world (this study - Canada and New Zealand; Ouchiyama *et al.*, 1993 - Japan).

In the creosote-contaminated Borden aquifer, carbazole was detected in the contaminated groundwater as far as 8 m from the source 471 days after the emplacement of creosote, and is moving through the contaminated plume at about the same rate as naphthalene (Fowler *et al.*, 1994). The portion of the aquifer in which carbazole has been detected has become anaerobic, and thus carbazole would not be subject to aerobic biodegradation in this aquifer. To date, carbazole biodegradation under anaerobic conditions has not been demonstrated. However, an *in situ* treatment technique, "funnel-and-gate" is being proposed for this portion of the aquifer (Fowler *et al.*, 1994) in which oxygen and nutrients will be added so that aerobic biodegradation can take place. Under these conditions, carbazole may be biodegraded by microorganisms in the aquifer to some of the products identified in this project.

In this study, carbazole loss (mineralization) was observed in the presence of a complex mixture of chemicals (Norman Wells crude oil, Section 5.3.4.3) and carbazole was also removed by mixed populations when it was added to commercial coal tar creosote (Chapter 3, Section 3.3.2.3.2). Thus, carbazole has the potential to be biodegraded within a complex mixture of contaminants under aerobic conditions, although whether this actually occurs under field conditions remains to be seen.

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6. OVERALL DISCUSSION, SUMMARY, AND SUGGESTIONS FOR FUTURE RESEARCH

Coal tar creosote, a widely used wood preservative, has been found to contaminate soils and groundwaters at several sites in Canada, the United States, and Europe. Creosote is a complex mixture of chemicals consisting of phenols, PAHs, and NSO compounds, many of which have been found to be toxic, teratogenic, and/or carcinogenic (Enzminger and Ahlert, 1987). The presence of toxic creosote chemicals in groundwater poses a threat to public health, especially if contaminated groundwater serves as a primary drinking water source for an area. When creosote enters soil or groundwater, both non-biological and biological processes can influence the fate of this complex mixture of chemicals. It has become increasingly evident, however, that the biodegradation of creosote compounds contributes significantly to the losses of these compounds at contaminated sites (Mueller *et al.*, 1991). Given the proper environmental conditions and the genetic capabilites to biodegrade creosote compounds, microbial populations in soils and groundwaters can serve as useful biocatalysts to remediate contaminated sites. Bioremediation, a treatment technology which exploits the abilities of microorganisms to biodegrade toxic chemicals to innocuous products, has received increased attention as a cost-effective method to remediate creosote-contaminated sites (Ryan *et al.*, 1991).

The development of bioremediation as a treatment technology has relied not only on experience at field sites, but also on the ever-increasing body of knowledge on the biodegradation of contaminant compounds as a result of laboratory studies. Before one can determine the environmental fate of contaminant compounds like those found in creosote, it is often necessary to understand the mechanisms by which these compounds are degraded (Liu *et al.*, 1992). For example, one of the most important steps in determining the feasibility of applying bioremediation to a contaminated site involves testing whether microorganisms are present that can biodegrade the contaminant(s) as well as the environmental conditions that are necessary for biodegradation to occur, such as the presence of oxygen (Bouwer, 1992). Laboratory studies, however, only indicate the potential for biodegradation of contaminants, and do not guarantee that biodegradation will occur in a complex field environment (Madsen, 1991).

Phenols, PAHs, and NSO compounds have been the subjects of hundreds of laboratory biodegradation studies under aerobic and anaerobic conditions using materials from contaminated sites, mixed laboratory cultures, as well as pure cultures of microorganisms, often isolated from contaminated materials (Berry *et al.*, 1987; Arvin *et al.*, 1989; Cerniglia, 1992). These studies can provide information about biodegrability of a compound, conditions under which a compound is biodegradable, metabolites that are produced, toxicity of a contaminant and its breakdown products, enzymes that are responsible for biodegradation, as well as genetic control of biodegradation pathways.

Many mechanisms of the biodegradation of aromatic compounds have been elucidated and generally accepted, although much is yet to be learned about the biodegradation of many compounds present in coal tar creosote under both aerobic and anaerobic conditions. For example, researchers have only recently identified microorganisms which are capable of growing on and biodegrading compounds in creosote generally thought to be recalcitrant, such as 4- and 5-ringed PAHs (Cerniglia, 1992), and with time, more compounds are being found to be biodegradable under anaerobic conditions. Much of this information, while not directly applicable to field sites, can be of use in predicting the fate of contaminant compounds in soil or groundwater, alongside other important chemical, physical, and hydrogeological studies of subsurface environments.

The research presented in this thesis consisted of laboratory studies examining three different aspects of creosote biodegradation as part of a larger research project examining the fate and attenuation of coal tar creosote compounds in a well-characterized aquifer

(Fowler *et al.*, 1994; King *et al.*, 1994). The findings of these studies are summarized below.

In the first of these studies, seven creosote-degrading populations were established which biodegraded creosote chemicals to different extents in the laboratory. It was found that the mixed populations (DT cultures) which had been enriched from creosote-contaminated soil contained bacteria which degraded creosote compounds to a greater extent than those that had been long maintained on crude oil (SL, ERN BIO, and ESSO AG) and that these cultures were capable of biodegrading all of the compounds in OCT that were monitored with the analytical methods used. The DT Mixed and Medium cultures were capable of biodegrading typically more recalcitrant 4-ringed PAHs such as fluoranthene, pyrene, benzo(a)fluorene, benzophenanthrene, and chrysene to a large extent, whereas the other five cultures generally only degraded the 2- and 3-ringed PAHs. As would be expected, the cultures degraded creosote compounds to a greater extent after 8 weeks than after 4 weeks of incubation. The DT Mixed culture was able to degrade several more creosote compounds at 10°C than the RMH Soil culture, which is likely a reflection of the different genetic capabilites of the two cultures. Upon screening the cultures for different PAH-degraders, all seven contained naphthalene- and phenanthrene-degraders, whereas only the RMH Soil and DT cultures contained anthracene- and pyrene-degraders. The DT enrichment cultures could prove useful as sources of HMW PAH-degraders for use in bioreactors to treat creosote-contaminated materials but more characterization of the populations would be necessary, such as determining whether the organisms could biodegrade or biotransform compounds containing 5 or more aromatic rings. If nothing else, the creosote-degrading populations established here could serve as sources of pure microorganisms for the metabolism of PAHs. Measuring the relative toxicity of the culture supernatants before or after creosote degradation would be of interest for future studies to see if the loss of creosote compounds actually results in a decrease in the toxicity of this material.

Also, some preliminary experiments were carried out to determine if the Borden aquifer sediments had iron-reducing activity. When a shallow aquifer becomes contaminated with organic chemicals, various redox zones generally develop with a methanogenic zone near the contaminated source and an aerobic zone at the leading edge of a contaminant plume. Because creosote is complex mixture of aromatic compounds with varying water solubilites, individual contaminants will migrate away from the source along with the groundwater at various rates depending on their water solubilities. At the Borden aquifer site, for example, which has been contaminated with creosote for just over four years, phenols have essentially disappeared from the source and are no longer detected in the contaminant plume, more water-soluble N-heterocycles like quinoline and indole have migrated to the outer edges of the contaminant plume, whereas other compounds like naphthalene and carbazole are moving slower and are closer to the source (Fowler *et al.*, 1994). Much of the groundwater in this creosote-contaminated aquifer is anaerobic, and it is thus important to determine whether compounds in the various zones are amenable to anaerobic biodegradation.

The Borden aquifer was considered to have an abundance of Fe(III) which could feasibly be used as a terminal electron acceptor for biodegradation of creosote compounds. Material sampled from aerobic and anaerobic zones of the Borden aquifer were found to contain microbial iron-reducing populations, because an increase in Fe(II) production was observed when live sediments were incubated with several different carbon sources in laboratory microcosms in the presence of added $Fe(OH)_3$. This was not seen in Borden sand which was heat-killed. Only small increases in Fe(II) were observed when the aquifer material was incubated with different carbon sources in the absence of added Fe(III), but large increases in Fe(II) were seen when $Fe(OH)_3$ was added as a Fe(III) source. The addition of substrates such as glucose, lactate, acetate, propionate, butyrate, or benzoate generally stimulated iron-reduction activity in the sediments, but other aromatic compounds (except phenol in one case) did not. Iron-reducing activity was also observed when anaerobic sediments were incubated with water-soluble creosote compounds, which contains a mixture of phenols and LMW PAHs and N-heterocycles.

Although these results suggest that the Borden sediments may be capable of oxidizing organic matter and creosote-contaminants under iron-reducing conditions, many more studies are required to verify this. For example, substrate loss was not regularly monitored in these preliminary experiments, and corroborating substrate loss with an increase in Fe(II) production would be a stronger indication of organic compound oxidation under iron-reducing conditions. Furthermore, demonstrating substrate loss and Fe(II) production in sediment cultures which contain only naturally-present forms of Fe(III) would be a better indication of the amount of iron-reducing activity that would actually be occurring in the Borden aquifer, as would experiments in which the cultures are incubated at more realistic temperatures (such as 10°C instead of room temperature). Other experiments which might be useful include monitoring the growth of sediment populations, monitoring the loss of Fe(III) and the identification of Fe(III), Fe(II) and/or mixed Fe(III)-Fe(II) minerals that may be forming as a result of microbial iron-reducing activity. The relative importance of the biodegradation of creosote components under iron-reducing conditions at the actual field site would also have to be determined, but the presence of iron-reducing populations in the Borden aquifer may allow for the natural loss of some creosote compounds due to biodegradation under iron-reducing conditions.

In addition to biodegradation studies of several chemicals in creosote, the aerobic biodegradation of carbazole, a N-heterocycle commonly found in creosote, was studied. Indeed, this was the main focus of the research. Although some studies were initially carried out with mixed carbazole-degrading cultures, a pure carbazole-degrading bacterium, *Pseudomonas* sp. LD2, was isolated from creosote-contaminated soil and used to study carbazole metabolism. This organism used carbazole as a sole source of carbon, nitrogen, and energy, and was found to mineralize a significant portion of ^{14}C-carbazole to $^{14}CO_2$.

In an effort to elucidate the mechanism of carbazole biodegradation by isolate LD2, several aromatic compounds were tested as growth substrates. This carbazole-degrading isolate was unable to use most of the aromatic compounds tested, but was found to biotransform the carbazole analogs dibenzothiophene, dibenzofuran, and fluorene in the presence of carbazole. Ring cleavage products of dibenzofuran which had previously been reported in the literature as a result of angular dioxygenation were identified in LD2 culture supernatants (Strubel *et al.*, 1991) which suggested that isolate LD2 cleaved carbazole in a similar manner. Ten N-containing metabolites were consistently observed to be present in organic extracts of acidified LD2 culture supernatants after derivatization with diazomethane and GC analysis, several of which were newly identified compounds. Four of these were tentatively identified, two were unidentifiable, and four were positively identified based on comparison with authentic standards or NMR analyses. Many of these compounds were also found to be present in the supernatants of mixed carbazole-degrading cultures maintained throughout the course of this work. Several of the metabolites were presumed to contain 2-aminobenzoyl-type structures which suggested that carbazole was cleaved in an angular fashion by isolate LD2 as had been previously proposed (Ouchiyama *et al.*, 1993). Compound **X**, unequivocally identifed by several NMR techniques, was postulated to be an artifact resulting from the cyclization of an enzymatically-produced carbazole intermediate resulting from angular dioxygenation, and a mechanism for the formation of this newly-identified compound was proposed. The detection of indole-3-acetic acid, a newly identified carbazole metabolite, and the ability of isolate LD2 to grow on this compound and other indole derivatives suggested that another mechanism of carbazole degradation could be operating in isolate LD2.

The relative importance of the 2-aminobenzoyl-type compounds or indole-3-acetic acid as intermediates was not clear, so a definitive pathway for carbazole biodegradation could not be proposed with any certainty. Nevertheless, based on the identification of metabolites in this study, a pathway for the biodegradation of carbazole by isolate LD2 was proposed (Fig. 5.25). The unequivocal identification of anthranilic acid and *cis, cis*-muconic acid from carbazole degradation as well as the detection of catechol as a metabolite from anthranilic acid degradation confirmed the lower pathway for carbazole degradation as had been previously proposed (Ouchiyama *et al.*, 1993). Further research should be undertaken to elucidate the upper pathway of carbazole metabolism by this bacterium and define which compounds are actual carbazole intermediates and which are the products of side reactions.

As a part of a project to investigate the biodenitrogenation of fossil fuels, research is currently in progress at the University of Houston, Texas to isolate and characterize the enzymes which are responsible for carbazole degradation by isolate LD2, such as the presumed intial angular dioxygenase enzyme, as has been characterized for a dibenzofuran-degrading *Sphingomonas* sp. (Bunz and Cook, 1993). Although the LD2 mutants screened using the methods in this work did not provide any insights as to which compounds were transient carbazole intermediates, more in-depth studies with these and other mutants could help elucidate the carbazole pathway, expecially if mutants could be obtained which accumulated single carbazole degradation products. Studies to identify the genes responsible for carbazole degradation using LD2 mutants are also currently underway at the University of Houston and will hopefully aid in the understanding of carbazole degradation by this and other carbazole-degrading bacteria.

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APPENDIX A

MEDIA COMPOSITIONS

Liquid media for culturing of aerobic bacteria

Stanier's Mineral Medium

(modified from Bennett *et al.*, 1986)

NH_4Cl	1.0 g
K_2HPO_4	1.0 g
$MgSO_4{\cdot}7H_2O$	0.2 g
$FeSO_4{\cdot}7H_2O$	0.01 g
$CaCl_2$	0.01 g
H_2O	1.0 L
Trace Metals solution	1.0 mL

Trace Metals solution

(Fedorak and Grbic´-Galic´, 1991)

$CaCl_2{\cdot}2H_2O$	3.7 g
H_3BO_3	2.5 g
$MnCl_2$	0.87 g
$FeCl_3$	0.65 g
$ZnCl_2$	0.44 g
$Na_2MoO_2{\cdot}2H_20$	0.29 g
$CoCl_2$	0.01 g
$CuCl_2$	0.0001 g
H_2O	1.0 L

B+N

K_2HPO_4	0.5 g
Na_2SO_4	2.0 g
$MgSO_4{\cdot}H_2O$	0.2 g
KNO_3	2.0 g
NH_4Cl	1.0 g
$FeSO_4{\cdot}7H_2O$	trace
H_2O	1.0 L
Trace Metals solution	1.0 mL

B-N

K_2HPO_4	0.5 g
Na_2SO_4	2.0 g
$MgSO_4{\cdot}7H_20$	0.2 g
KCl	2.0 g
$FeSO_4{\cdot}7H_2O$	trace
H_2O	1.0 L
Trace Metals solution	1.0 mL

For these media, weigh out the appropriate amounts of all compounds except $FeSO_4{\cdot}7H_2O$ and dissolve, with stirring, in H_2O. When dissolved, add $FeSO_4{\cdot}7H_2O$ and trace metals and stir for at least 5 min to ensure that all compounds are dissolved. Dispense and autoclave.

Liquid media for culturing of iron-reducers

Wolfe's Vitamin Solution

(ATCC, 1993)

Biotin	2.0 mg
Folic acid	2.0 mg
Pyroxidine-HCl	10.0 mg
Thiamine-HCl	5.0 mg
Riboflavin	5.0 mg
Nicotinic acid	5.0 mg
Na Pantothenate	5.0 mg
Cyanocobalamine	trace
p-Aminobenzoate	5.0 mg
Thioctic acid	5.0 mg
ddH_2O	1.0 L

Mix all ingredients and store solution at 4°C.

Wolfe's Mineral Solution

(ATTC, 1993)

Nitrilotriacetic acid	1.5 g
$MgSO_4 \cdot 7H_2O$	3.0 g
$MnSO_4 \cdot H_2O$	0.5 g
NaCl	1.0 g
$FeSO_4 \cdot 7H_2O$	0.1 g
$CoCl_2 \cdot 6H_2O$	0.1 g
$CaCl_2$	0.1 g
$ZnSO_4 \cdot 7H_2O$	0.1 g
$CuSO_4 \cdot 5H_2O$	0.01 g
$AlK(SO_4)_2 \cdot 12H_2O$	0.01 g
H_3BO_3	0.01 g
$Na_2MoO_4 \cdot 2H_2O$	0.01 g
$NiCl_2 \cdot 6H_2O$	0.025 g
ddH_2O	1.0 L

Add nitrilotriacetic acid to 500 mL ddH_2O and adjust pH to 6.5 with NaOH, then add other ingredients in the order they are listed and bring volume up to 1.0 L. Store solution at 4°C.

Fe(III) Reduction Medium

(Lovley and Phillips, 1988)

$NaHCO_3$	2.5 g
$CaCl_2 \cdot 2H_2O$	0.1 g
KCl	0.1 g
NH_4Cl	1.5 g
$NaH_2PO_4 \cdot H_2O$	0.6 g
H_2O	980 mL
Wolfe's Vitamin solution	10 mL
Wolfe's Mineral solution	10 mL

Mix all ingredients together. Bubble 30%CO_2/70% N_2 through solution for at least 30 min before dispensing into serum bottles. Autoclave.

Fe(III) Citrate Medium

(Lovley and Phillips, 1988)

$FeC_6H_5O_7 \cdot 5H_2O$	17.0 g
$NaHCO_3$	2.5 g
KCl	0.1 g
NH_4Cl	1.5 g
$NaH_2PO_4 \cdot H_2O$	0.6 g
H_2O	980 mL
Wolfe's Vitamin solution	10 mL
Wolfe's Mineral solution	10 mL

Heat ferric citrate in 980 mL until dissolved. When cool, adjust pH to 7, then add rest of ingredients. Bubble with 30% CO_2/70% N_2 at least 30 min before dispensing into serum bottles. Autoclave.

Fe(III) Enrichment Medium

(modified from Caccavo *et al.*, 1992)

$FePO_4$	3.0 g
$NaHCO_3$	2.5 g
NH_4Cl	1.5 g
KH_2PO_4	0.6 g
H_2O	1.0 L
Trace Metals solution	1.0 mL

Allow $FePO_4$ to dissolve overnight. Add rest of ingredients, adjust pH to 7, then bubble with 30%CO_2/70% N_2 for at least 30 min before dispensing into serum bottles. Autoclave.

Liquid medium for methanogenic enrichments

Mineral Solution I

NaCl	5.0 g
$CaCl_2 \cdot H_2O$	1.0 g
NH_4Cl	5.0 g
$MgCl_2 \cdot 6H_2O$	1.0 g
0.01 M HCl	100 mL

Mineral Solution II

$(NH_4)_6Mo_7O_{24} \cdot 2H_2O$	1.0 g
$ZnSO_4 \cdot 7H_2O$	0.01 g
H_3BO_3	0.03 g
$FeCl_2 \cdot 4H_2O$	0.15 g
$CoCl_2 \cdot 6H_2O$	0.003 g
$NiCl_2 \cdot 6H_2O$	0.003 g
$AlK(SO_4)_2 \cdot 12H_2O$	0.01 g
H_2O	100 mL

Phosphate Solution

5.0 g KH_2PO_4 per 100 mL H_2O

Resazurin Solution

0.01 g per 100 mL H_2O

WR86 Medium

(Fedorak and Westlake, 1984)

Mineral Solution I	1.0 mL
Mineral Solution II	0.1 mL
Resarurin Solution	1.0 mL
Phosphate Soution	1.0 mL
$NaHCO_3$	0.57 g
H_2O	100 mL

Mix together all but $NaHCO_3$. Boil for 5 min, then cool on ice while bubbling with 30%CO_2/N_2. Add $NaHCO_3$ when cool, and bubble till pH is 6.9-7.1 (20 min) before dispensing into serum bottles. After autoclaving, reduce with 2.5% w/v Na_2S, at rate of 1 mL/100 mL.

Agar plates

Carbazole Medium

(modified from Ouchiyama *et al.*, 1993)

Na_2HPO_4	2.2 g
KH_2PO_4	0.8 g
KCl	2.0 g
$CaCl_2$	0.015 g
$MgSO_4$	0.03 g
$FeSO_4 \cdot 7H_2O$	trace
Trace Metals solution	1.0 mL
H_2O	1.0 L
Difco agar	15.0 g

Combine all ingredients and autoclave. Prepare a solution of 0.5 g carbazole in 20 mL dimethylsulfoxide. When hot medium has cooled to about 50°C, add carbazole solution, swirl, and pour plates.

BYP with Kanamycin

B+N	1.0 L
Yeast extract	1.0 g
Proteose peptone 3	1.0 g
Difco agar	15.0 g

Combine all ingredients and autoclave. When agar has cooled to about 50°C, add 100 µg/mL kanamycin and swirl before pouring plates.

Mineral Medium plates

K_2HPO_4	4.4 g
KH_2PO_4	1.7 g
NH_4Cl	2.1 g
Salt solution*	10 mL
H_2O	990 mL
Difco agar noble	15 g

*** Salt solution** - filter sterilize and use sterile pipet when adding to medium

$MgSO_4 \cdot 7H_2O$	19.5 g
$MnSO_4 \cdot H_2O$	5.0 g
$FeSO_4 \cdot 7H_2O$	5.0 g
$CaCl_2 \cdot 2H_2O$	0.3 g
Ascorbic acid	1.0 g
H_2O	1 L

SOURCES OF CHEMICALS USED

All chemicals (excluding inorganic salts used for media) used in cresosote, carbazole, and iron reduction studies are listed, alphabetically, along with the company from which they were obtained. All ^{14}C-labelled compounds were from Amersham Corp. (Arlington Heights, Illinois). Organic solvents used for extractions and HPLC analyses, including dichloromethane, ethyl acetate, acetonitrile, and methanol, as well as acids like HCl, H_2SO_4, and CH_3COOH were obtained from different sources over the years.

Chemical	**Source**
Acetic anhydride	Fisher Scientific Co. (Fair Lawn, NJ)
ACS Fluor	Amersham
2-Aminobiphenyl	Aldrich (Milwaukee, WI)
Anthranilic acid	BDH (Poole, England)
Benzene	Fisher Scientific Co.
Benzoic acid	Fisher Scientific Co.
Biphenyl	Eastman Kodak Co. (Rochester, NY)
N,*O*-bis-(trimethylsilyl)acetamide	Pierce Chemical Co. (Rockford, IL)
Boron trifluoride-methanol	Aldrich
Carbazole	BDH
Carbo-Sorb II	Packard Instruments (Illinois)
Catechol	Fisher Scientific Co.
Chloramphenicol	Sigma (St. Louis, MO)
Chloroform (deuterated)	Norell Inc. (Landisville, NJ)
Dibenzofuran	Aldrich
Dibenzothiophene	Fluka (Ronkonkoma, NY)
2,2'-Dihydroxybiphenyl	Sigma
2,3-Dihyroxybiphenyl	Wako Pure Chemicals (Osaka, Japan)
N,*N*-Dimethylformamide	Sigma
Ferrozine	Sigma
Fluorene	Aldrich
Glucose	Sigma
HEPES	ICN Biomedicals (Aurora, OH)
n-Hexadecane	Fisher Scientific Co.
2-Hydroxycarbazole	Aldrich
Iminostilbene	Aldrich
Indole	Matheson, Coleman & Bell (Norwood, OH)
Indole-3-acetic acid	Sigma
trans-Indole-3-acrylic acid	Aldrich
Indole-3-butanoic acid	Aldrich
Indole-3-carboxaldehyde	Aldrich
Indole-3-carboxylic acid	Aldrich
Indole-3-propanoic acid	Aldrich
Indole-3-pyruvic acid	Sigma
Kanamycin	Sigma
DL-Kynurenine	Sigma
N-Methylcarbazole	Aldrich
N-Methyl-*N*-nitroso-*N*-nitroguanidine	Aldrich
trans,trans-Muconic acid	Aldrich
Naphthalene	Sigma

Chemical	**Source**
2-Nitrobiphenyl	Aldrich
Peracetic acid (13.5%)	Aldrich
Phenanthrene	Aldrich
Phenol	Mallinckrodt (Montreal, Quebec)
Phenothiazine	Aldrich
Plate count agar	Difco (Detroit, MI)
Pyridine	Raylo Chemicals (Edmonton, Alberta)
Sodium acetate	BDH
Sodium benzoate	BDH
Sodium periodate	Matheson, Coleman & Bell
Sodium pyruvate	Sigma
Sodium succinate	Fisher Scientific Co.
1,2,3,4-Tetrahydrocarbazole	Aldrich
Trypic soy broth	Difco
L-Tryptophan	Sigma

APPENDIX B

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

All NMR experiments and interpretation were done by Dr. Albin Otter in the Department of Chemistry, University of Alberta.

NMR Methods

All NMR spectra were recorded on a Varian Unity 500 spectrometer with a Varian 5 mm z-gradient inverse-detection triple probe (^1H and inverse detection experiments) and a 5 mm broad-band probe (APT experiment) (Patt and Shoolery, 1982). VNMR software version 4.3A was used for data acquisition and processing. The sample concentrations were approximately 0.3 mM (compound **VI**) and 6 mM (compound **X**) in $CDCl_3$. All spectra were recorded under temperature controlled conditions at 30.0 ± 0.1°C. Chemical shifts are reported relative to the solvent peak at 7.24 ppm (^1H) and 77.0 ppm (^{13}C) downfield from TMS. Two-dimensional spectra (Patt and Shoolery, 1982) were recorded non-spinning.

Gradient-enhanced correlation experiments (GCOSY) (Hurd, 1990) were carried out as described elsewhere (Otter *et al.*, 1995). The Heteronuclear Multiple Bond Coherence (HMBC) experiment (Bax and Summers, 1986) was recorded with a 5,000 Hz sweep width/4K data points (no zero-filling) in the proton dimension and 20,000 Hz sweep width/400 (zero-filled to 1K) data points in the ^{13}C dimension. Thirty-two scans were accumulated per t_1-increment and the data were multiplied prior to Fourier transformation by cosine functions of width one half of the acquisition time in t_2 and t_1, respectively. Data were recorded in the phase-sensitive mode (States *et al.*, 1982) but presented in so-called mixed mode (Bax and Marion, 1988), whereby phase-sensitivity is retained in the F2-dimension while absolute value mode is used in F1. A homospoil pulse preceded a 1.1 sec relaxation delay. BIRD ^{12}C-signal suppression (Bax and Subramanian, 1986) was used with a 0.5 sec delay and directly coupled signals were suppressed with a delay based on a 150 Hz ^1J carbon/proton coupling constant. The multiple bond delay was set to 70 msec.

A Heteronuclear Multiple Quantum Coherence (HMQC) experiment (Bax and Subramanian, 1986) was recorded with similar parameters as described above for the HMBC with no broadband decoupling of the ^{13}C domain to obtain the direct carbon/proton coupling constants.

The APT (Attached Proton Test) spectrum was recorded with 65K (zero-filled to 128K) data points over a spectral range of 25,000 Hz. Over 27,000 transients were accumulated with a relaxation delay of 1.3 sec. The APT-specific delays (Patt and Shoolery, 1982) were $t = 7$ msec and $\Delta = 1$ msec and composite pulses were used for the two 180° pulses in the sequence. 1.0 Hz line-broadening was applied prior to Fourier transformation and phasing was chosen so that C and CH_2 signals are on the same side of the spectrum as the residual solvent signal and CH and CH_3 of opposite sign.

NMR Data Interpretation

Compound VI

The following chemical shifts and coupling constants in $CDCl_3$ solution at 30.0 °C (numbers are used for the quinoline ring system, a_{cis} and a_{trans} for the protons of acetic acid relative to H2): H1 = NH: 1.60 ppm, very broad; H2: 4.03 ppm, d x d x d x d (16 lines): 11.1, 10.2, 4.3, 3.2 Hz; H3(ax): 2.68 ppm, d x d: -16.7, 10.2 Hz; H3(eq): 2.56 ppm, d x d: -16.7, 3.2 Hz; Ha_{cis}: 2.71 ppm, d x d: -16.1, 4.3 Hz; Ha_{trans}: 2.51 ppm, d x d: -16.1, 11.1 Hz; H5: 7.81 ppm, d x d: 7.9, 1.7 Hz; H6: 6.74 ppm, d x d x d: 7.9, 7.1, 1.0 Hz; H7: 7.30 ppm, d x d x d: 8.2, 7.1, 1.7 Hz ; H8: 6.68 ppm, d x d: 8.2, 1.0 Hz. Based on the very large a_{cis}/a_{trans} geminal coupling constant and the large vicinal coupling between H2 and Ha_{trans}, it appears very likely that the carbonyl group of the acetic acid residue is hydrogen bonded to the amine proton thereby forming a six-membered ring.

Such large coupling constants are usually not found in saturated, unrestricted chain structures but are consistent with a six-membered ring.

Compound X

Compound **X** was unequivocally identified as 3a,4-dihydro-2-methoxy-pyrrolo[1,2-a]quinoline-1,5-dione based on one- and two-dimensional ^1H and ^{13}C-NMR spectroscopy. The results are summarized in Table B.1.

Based on proton NMR, one methyl group plus two distinct spin systems of four protons each were identified with no homonuclear coupling between the two systems. One set was clearly of aromatic nature (H6 to H9) whereas the other one consisted of one proton attached to a double bond (H3) and three saturated protons (H3a, H4 axial and equatorial). In the ^{13}C domain, the APT spectrum (Fig. B.1) revealed the presence of 5 quaternary carbons and one CH_2 group. The large geminal coupling constant (-16.0 Hz) and particularly the very large coupling of 14.0 Hz between two vicinal protons suggested, as for compound **VI** before, the presence of a six-membered ring because neither open chains nor smaller or larger ring structures would entail coupling constants of this magnitude.

The assignment of the protonated carbons was straightforward based on a two-dimensional HMQC experiment. Nevertheless, the question of how to link the two 4-spin systems together remained a difficult task, especially because neither of the protons of each spin system was close enough in space to any proton of the other spin system to yield any appreciable Nuclear Overhauser Effects (NOEs) in one- and two-dimensional rotating-frame cross-relaxation experiments (T-ROESY, data not shown) (Hwang and Shaka, 1993). However, all uncertainties could be eliminated by recording a two-dimensional HMBC spectrum (Fig. B.1). In short, this technique correlates protons and carbons which are not directly linked by a heteronuclear ^1J coupling constant but via *multiple* bonds (mainly ^3J but also ^2J). As can be seen in Fig. B.1, a large number of correlations was

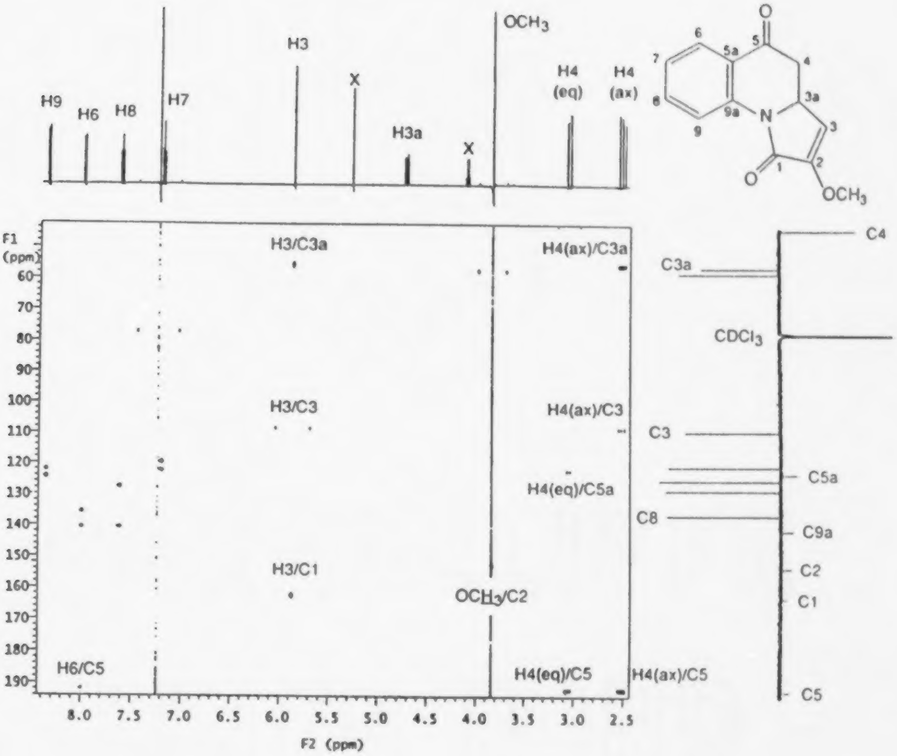

Figure B.1 The two-dimensional HMBC spectrum of compound **X** in $CDCl_3$ solution together with the one dimensional proton spectrum (top) and the APT spectrum (right side). Impurities of the sample are denoted with an x. The two vertical ridges are due to incomplete elimination of the intense ^{12}C signal of the solvent and the methoxy group. Direct response correlation peaks (through one bond coupling) are visible for the aforementioned signals as well as H3. Experimental details are given in the text and NMR data are summarized in Table B.1.

Table B1: ^{1}H and ^{13}C NMR data of compound **X** in $CDCl_3$ solution at 30.0 °C [a, b]

	1	2 [c]	3	3a	4	5	5a	6	7	8	9	9a
$\delta(^1H)$	--	--	5.87	4.73	2.52 (ax) 3.05 (eq)	--	--	8.00	7.20	7.62	8.36	--
$^3J(^1H^1H)$	--	--	2.4	2.4 14.0 3.5	d 14.0 (ax) 3.5 (eq)	--	--	7.9	7.4	8.4 7.4	8.4 7.9	--
$^4J(^1H^1H)$	--	--	--	--	--	--	--	1.7	1.2	1.7	1.2	--
$\delta(^{13}C)$	162.4	152.6	108.4	55.8	43.5	191.9	122.2	127.6	124.3	135.7	119.8	140.6
$^1J(^{13}C^1H)$	--	--	176.8	144.2	128.0 (ax) 136.0 (eq)	--	--	165.0	164.4	161.8	169.1	--

[a] Chemical shifts are reported in ppm relative to the solvent peak at 7.24 ppm (^{1}H) and 77.0 ppm (^{13}C) downfield from TMS. Coupling constants are listed in Hz.

[b] Compound **X** is numbered in accordance with Chemical Abstract Services (1988).

[c] The data for the 2-methoxy substituent are: 3.84 ppm (^{1}H) and 57.5 ppm (^{13}C) with a $^1J(^{13}C^1H)$ of 145.7 Hz.

[d] The geminal coupling constant is -16.0 Hz.

observed. The sequence of correlations, H6/C5, C5/H4(ax/eq) and C5a/H4(eq), clearly revealed the connection between the aforementioned spin systems. Interestingly, and maybe surprising at first, no correlations were observed between H3a and any of the surrounding carbon atoms. A computer analysis of an energy-minimized model of compound **X** revealed that the dihedral angles between H3a and the carbon atoms three bonds away (C5, C2 and C9a) were all within ± 20° of 80° at which the magnitude of the coupling constants, and hence the intensity of correlation peaks, is at a minimum (Bystrov, 1976). Given the angular dependence of the coupling constants, the absence of correlation peaks to H3a was, in fact, further proof that compound **X** was indeed the pyrrolo[1,2-a]quinoline-1,5-dione structure with the position of the methoxy group firmly established by the ^3J heteronuclear correlation to C2.

APPENDIX C

PREPARATION OF FE(II) STANDARDS FOR FERROZINE ASSAY

- adapted from Standard Methods for the Examination of Water and Wastewater, 17th Ed., 1989

Slowly add 20 mL concentrated H_2SO_4 to 50 mL H_2O in a beaker and dissolve 1.404 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$. Quantitatively transfer to a 100 mL volumetric flask, bring volume up to 100 mL and mix well. This is a 200 mg/L standard Fe(II) stock solution. Make dilutions of this stock as follows:

mL from stock	mg Fe(II)/100 mL	µg Fe(II) for 0.1 mL assay	µg Fe(II) for 1.0 mL assay
0	0	0	0
2	4	4	40
5	10	10	100
10	20	20	200
15	30	30	300
20	40	40	400
25	50	50	500

Prepare diluted stocks in 100 mL volumetric flasks. For ferrozine assay, add either 0.1 mL or 1.0 mL to test tubes containing 5 mL 0.5 N HCl to assay for appropriate amounts of Fe(II) as listed above. Store solutions at 4°C.

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